

The complex realities of sharing genetic assets

Debates on bioprospecting tend to be dominated by historical distrust and visions of new riches. Companies and countries alike need to recognize each other's needs, but remain realistic about the issues at stake.

Bioprospecting, gene-hunting, biopiracy: the terms used to describe the search for natural products of potential value to industry, particularly in the pharmaceutical and agri-business fields, reflect the spectrum of emotions that the practice generates.

Parts of this spectrum will be on display next month, when signatories to the UN Convention on Biodiversity, signed at the Earth Summit in Rio de Janeiro in 1992, hold their fourth meeting at Bratislava in Slovakia. Unsurprisingly, given its origins in the environmentalist concerns of the 1980s, the convention embodies sympathy for the argument that one of the biggest threats to environmental conservation is unconsidered rapaciousness by the private sector. Indeed, its direct challenge to free-market economics — expressed, in particular, through its acknowledgement of governments' sovereign rights over their natural resources — is a significant reason why the convention has not yet been ratified by the US Senate.

A different palette of the same emotions will be on display in 1999, when representatives of the world's economic ministries meet to discuss revisions to the so-called agreement on Trade Related Aspects of Intellectual Property (TRIPs), part of the negotiations that led to the setting up of the World Trade Organization in 1995. Here the assumptions are very different, namely that excessive government control on resources is to be avoided as an obstacle to economic growth. And such obstacles include the very constraints on the ability of individuals to negotiate market prices of biological resources that the biodiversity convention would allow.

As the Briefing in this issue demonstrates (see pages 535–540), current debates about bioprospecting are considerably more complex than just a confrontation over economic philosophies. There are, for example, widely differing views of the potential value of the assets at stake. Those who perceive a rich seam of gold ready to be mined seem likely to be disappointed. The pickings obtained so far have proved relatively meagre, while the increasing ability of combinatorial chemistry to provide an alternative and considerably more convenient route to novel molecules will only skew the ratio of costs to benefits still further against bioprospecting.

Nuggets

The nuggets to be found most probably reside in the genetic sequences of plants. Unpublished research at the Science Policy Research Unit (SPRU) at the University of Sussex, England, underlines the importance that industry is already attaching to intellectual property rights on plant DNA sequences. This has revealed a predictably high number of patents filed on major crops such as maize. Rather less predictable is the wide range of patents for DNA sequences already applied for on crops in developing countries that include coconut, the moth bean, nutmeg, coriander, castor bean and cocoa.

The SPRU researchers suggest that the rate of patents filed for plant DNA will rise rapidly over the next 10 to 15 years, as functional analysis of plant genomes creates unprecedented opportunities for crop improvement. Such developments can only increase sensitivi-

ties about questions of ownership, and in particular about the meaning of 'national sovereignty' over resources that is enshrined in the biodiversity convention. Similar questions will inevitably arise as the genomic sequencing of substances used in traditional herbal remedies reveals knowledge about their mode of functioning. Should the commercial benefit obtained from this knowledge accrue to those who discovered an active ingredient through a long process of trial and error? Or to the biotechnology company able to identify — and improve on — this key ingredient in precise scientific terms?

Dangers

Those who argue in favour of the first approach clearly hold the higher moral ground. Inspired by the successful battles by indigenous communities worldwide for compensation for the expropriation of their lands (and the mineral rights that went with them), such individuals and groups make an appealing case in favour of a socially equitable solution to the distribution of benefits. The danger, their critics point out, is when one man's equity becomes another man's barrier to efficient trade; protectionism is never far below the surface of the arguments of those who complain of exploitation by outsiders.

But there are dangers on the other side too. The strict application of patent laws which, by their nature, tend to favour the most scientifically advanced and innovative economies, place at a significant disadvantage those lacking such resources and skills. The TRIPs agreement would work well if scientific and technical skills were better distributed around the globe; it certainly provides an incentive to countries to develop such skills in order to become effective players in the global economy. But, if left unmodified on the questions of indigenous knowledge and community rights — two concepts without status in free-market economics — it risks enhancing social disparities and thereby fostering social conflict.

There are no easy routes to a resolution. But flexibility and negotiation are the watchwords. For example, companies could agree to buy exploration licences and reach agreement on profit sharing, on the understanding that the benefits to the country concerned should take the form of a direct contribution to its ability to analyse scientifically and make use of its biological resources. Some companies, ranging from the giant Merck to the small California-based Shaman Pharmaceuticals, are already experimenting in this direction. So too are projects such as the International Cooperative Biodiversity Group (ICBG), an expanding network of bioprospecting projects.

The science of genomics may be low on the priorities in some developing countries, while, given current tight research budgets, some companies are unlikely voluntarily to deliver technical assistance windfalls. In the long run, however, only solutions crafted on mutualities of interest — perhaps in the form of corporate support for an international initiative to promote biotechnology in developing countries, modelled on projects such as the ICBG and the International Centre for Genetic Engineering and Biotechnology — can overcome the mistrust and suspicion that dogs the current debate. □



Ex-UN AIDS chief is blasted for remarks on vaccine strategy

AP/TIM SHARP

[WASHINGTON] The former director of the World Health Organization's Global Programme on AIDS, Jonathan Mann, has accused the US National Institutes of Health (NIH) of violating human rights by failing to proceed to large-scale clinical trials of AIDS vaccines.

His remarks were made in an address to the President's Advisory Council on AIDS (PACHA) last month shortly before the council proposed that leadership of AIDS vaccine development be moved out of NIH and into the White House Office of National AIDS Policy.

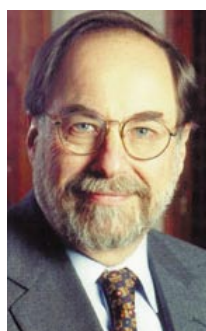
But leaders of NIH's effort to develop a vaccine argue that the remarks by Mann, who is now dean of the School of Public Health at Allegheny University of the Health Sciences in Philadelphia, are scientifically uninformed and inject politics unnecessarily into what should be a rational scientific debate.

"It's time that Dr Mann sits down with the data and looks at it," says David Baltimore, president of the California Institute of Technology, and head of a committee that advises NIH on AIDS vaccine research. "He should be involved in a scientific debate, not a political and rhetorical one."

Such sentiments are shared by Anthony



Mann (left) has irked Baltimore (right) and others with his criticism of NIH.



Fauci, the director of the National Institute of Allergy and Infectious Diseases: "It's unfortunate that Jonathan has put the difficulties that one encounters in vaccine development, and decisions about vaccine trials into the arena of human rights."

Addressing PACHA last month, Mann accused Baltimore and Harold Varmus, the NIH director, of incompetence and ethical failure because NIH has not launched large-scale phase III clinical efficacy trials of any HIV vaccine candidate.

After their safety and immunogenicity have been demonstrated, "vaccine candi-

dates are ready to be tested in human trials", he said. Therefore "the federal government's failure to proceed to vaccine field trials is a human rights violation".

Mann claimed that Baltimore, a virologist and molecular immunologist who won the Nobel prize in 1975 for his co-discovery of reverse transcriptase, is "not equipped to develop an AIDS vaccine" and that, with Varmus, he is "holding a monopoly lock on the process".

"This is too big a problem to leave scientists in charge of," Mann said, arguing that the ten-member committee that Baltimore heads — and which is dominated by basic scientists — should feature equal representation by vaccinologists and public health experts.

Mann, a medical doctor with a master's degree in public health, charged that, because AIDS disproportionately affects the poor and minorities, vaccine development is progressing more slowly than it would if the affluent were primarily afflicted. He also argued that it is "unrealistic and illusory" to wait for scientific consensus on when a vaccine candidate is ready, pointing out that scientists had been in conflict before they launched field trials of vaccines such as polio.

Mann is not alone in his assessment of NIH's vaccine work. "The current federal AIDS vaccine effort is stalled in paralysing scientific debate and bureaucratic delay," PACHA said in a statement three days after his address. "It is only within the Office of the President that sufficient authority exists to ensure leadership, thorough coordination and collaboration of all requisite constituencies."

But Mann's speech has piqued NIH leaders, who say he is ignoring the scientific fact that no vaccine candidate is ready for clinical efficacy trials. They point, for example, to a recent study, reported in the *Journal of Virology*, of 16 individuals who had become infected in phase I and phase II trials, despite vaccination with a gp120 vaccine, which relies for immunogenicity on a single subunit protein of HIV.

This paper concluded that the vaccine conferred no beneficial or adverse effects on the individuals. "There's an enormous amount of evidence here that the kinds of antibodies induced in vaccinees are not antibodies that can neutralize a virus, that can protect people," says Baltimore.

Baltimore says that Mann's arguments rely on generalities and historical analogies that do not pertain in the AIDS context, and when individual AIDS vaccine candidates are scrutinized.

South African drugs agency to be replaced

[CAPE TOWN] South Africa's Health Minister, Nkosazana Zuma, announced last week that the Medicines Control Council (MCC), which has clashed with the government by consistently blocking trials of a controversial AIDS drug, will be replaced by a new Medicines Regulatory Authority.

The move follows clashes between government and the MCC about the drug Virodene. In January last year, three researchers at the University of Pretoria claimed to have found a cure for AIDS and appealed directly to cabinet for funds for further research. But the following month the MCC issued a report outlining the dangers of the drug, and halted the illegal clinical trials that were already taking place (see *Nature* 385, 474 & 386, 6; 1997).

In January this year, the council again refused an application for clinical trials of Virodene, on the grounds that the drug contains a highly toxic industrial solvent, dimethylformamide, and that measurements to assess its effects had not been adequately developed.

Early last month the general secretary of the African National Congress (ANC), Kgalema Motlanthe, accused the MCC of

"censoring" research on the drug. A week later, the deputy president Thabo Mbeki took the extraordinary measure of launching a stinging attack on the council. In an article written for two of the country's Sunday newspapers, Mbeki concluded that "the cruel games of those who do not care (about AIDS) should not be allowed to set the national agenda".

Mbeki also vehemently denied claims by the opposition Democratic Party that the ANC had been offered a six per cent financial stake in the company that has patented the drug, Cyropreservation Technologies.

The heads of the departments of medicine at five of the country's seven medical schools have expressed great concern at "political interference" in the functioning of the MCC. In a signed letter, the academics expressed support for the council, which they felt had "served the public of South Africa with the highest degree of integrity".

Zuma's decision to create a new authority follows the recommendation last month of a report on the council by an independent evaluation team.

Michael Cherry

► However, Mann is unapologetic. The consternation he has stirred up at NIH is “appropriate”, he says. “I continue to fail to see the kind of clear, milestone-driven process [at NIH] that includes not only the opinions of basic scientists, but the knowledge and experience of people who have developed vaccines.”

His critics include Gregg Gonsalves, a spokesman for Treatment Action Group, a national AIDS research advocacy organization, who calls Mann’s speech “outrageous and irresponsible”. “There is this notion that there are these big bad NIH scientists standing in the way of phase III development of an effective HIV vaccine,” says Gonsalves. “The real story is that we don’t have an acceptable candidate to put into phase III trials right now.”

Varmus declines to comment on Mann’s

statements. But Fauci, whose institute carries out most of NIH’s AIDS work, called the statements “untrue” and said it was “inappropriate” for Mann “to personally attack Harold Varmus and David Baltimore”.

In 1994, Fauci decided that NIH should not back phase III trials of two gp120 vaccines (see *Nature* 369, 593; 1994). No other candidate has since been moved into such trials by NIH. But in January, VaxGen, a biotechnology company in South San Francisco, announced that it had gained the approval of the Food and Drug Administration to launch this year a privately funded phase III trial of gp120 vaccine in the United States and Thailand (see *Nature* 391, 220; 1998).

As chair of the NIH’s 15-month-old AIDS Vaccine Research Committee, Baltimore has been meeting scientists, studying candidate

vaccines, and developing and awarding ‘innovation’ grants to encourage scientists to work in areas that are not being adequately explored. “There’s a very extensive programme designed to understand and produce a vaccine,” he says.

Baltimore says that progress on the vaccine is not being limited by NIH’s unwillingness to test new candidates, “but by the limited number of opportunities being presented by the scientific community”. He adds: “It’s not necessarily anybody’s fault. It’s a very difficult problem.”

Gonsalves rejects the advisory council’s proposal that control of AIDS vaccine development be moved to the White House: “It’s a scientific problem. Our best resources, our best talent are at the NIH. And that’s where the job should be done.” **Meredith Wadman**

New Chinese premier takes on the reins of scientific reform

[TOKYO] China’s new and outspoken premier, Zhu Rongji, seems likely to champion science and technology. He has already taken personal charge of making increased support for science a reality, a measure promised by his government.

In his first press conference after his appointment at the end of last month, Zhu lambasted the waste of government money in what he called an “excessive” number of government bodies and projects, and complained that this has been depriving science and technology of necessary funds.

Zhu, a tough and talented reformer, is believed to be the principal architect of recent reforms that have reduced the number of state ministries and commissions from 40 to 29, and have also resulted in the State Science and Technology Commission being converted into a ministry (see *Nature* 392, 220; 1998).

Furthermore, he will chair a powerful, newly reconstituted committee for science, technology and education — its forerunner was restricted to science and technology alone — in the State Council, the highest body for the day-to-day running of the country, which is headed by Zhu himself.

A senior member of the Chinese Academy of Sciences says he “applauds” the determination and drive of the new premier, and predicts that Zhu’s committee will play a “crucial” role in implementing policy and revitalizing China through science and technology.

He is also optimistic that Zhu will push forward a promised government programme to pump 2.5 billion yuan (US\$300 million) into basic research over the next five years, although he points out that the definition of “basic research” remains “somewhat controversial” in China.

The new government has already said that it plans to increase spending on science



Zhu Rongji: the premier’s committee will be crucial for implementing science policy.

this year by 12.6 per cent, to a total of 12.5 billion yuan. But the head of the newly formed Ministry of Science and Technology, Zhu Lilan, a polymer chemist, seems likely to emphasize development more than basic research.

In an interview with the *People’s Daily* newspaper on 30 March, she pointed out that in developed countries expenditure on basic research compared with development and commercialization falls into the ratio of 1:10:100. But in China the ratio is about 1:0.5:100.

“We need to look closely at the conditions and environment that slow down the transfer of scientific and technical results to industry,” the newspaper quotes Zhu Lilan as saying. She added: “As a developing country, we need to concentrate our resources in order to make strategic breakthroughs. We can’t afford to scatter our investments thoughtlessly.”

But the new science minister also calls for more creativity and innovation. “We need to create an environment in which young people can step forward from the crowd and sing their own song.”

It remains unclear what effect the conversion of the State Science and

Technology Commission to a ministry will have. On paper, stepping down from the State Council reduces its status. But it is expected to continue to play a leading role in setting science and technology policy, and as a ministry it will be able to fund its own research, rather than channelling funding through other ministries.

However, Western observers in Beijing point out that changes in central government alone will be insufficient to bring about drastic reform, as in China considerable power and budgets are wielded by provincial governments. Changes mirroring the reform of central government are not expected in the provinces until next year, and it remains to be seen whether they will fully implement the reforms desired by leaders in the central government.

Furthermore, one of the biggest problems facing China’s research system is the lack of coordination between different branches of the government, both central and provincial. One factor holding back the development of the Internet for scientific use in China, for example, is the lack of good connections between the network of the Chinese Academy of Sciences and the computer network for the universities.

Meanwhile, the revamped Ministry of Science and Technology has appointed its former vice-minister, Huang Qitao, previously director of the State Bureau for Nuclear Safety under the State Science and Technology Commission, to head its investment arm, China Venturetech Investment Corporation.

Officials quoted in the *South China Morning Post* newspaper say that although the appointment of a former vice-minister did not necessarily mean an upgrade for China Venturetech, the move was likely to sharpen the company’s teeth in the long run.

David Swinbanks

Call to scrap licences for travel to Cuba

[WASHINGTON] Stringent travel rules are effectively blocking scientific collaboration between the United States and Cuba, and should be applied more fairly or scrapped altogether, according to a report from the American Association for the Advancement of Science (AAAS).

A meeting held in Washington DC last week to discuss the report was told of sometimes "arbitrary and capricious" granting of licences to spend money on travel to Cuba by the Department of the Treasury's Office of Foreign Assets Control (OFAC).

Despite its struggling economy, Cuba is the most scientifically advanced of the Caribbean islands, say AAAS officials. For example, Fidel Castro's communist government has invested \$1 billion since 1980 in a well-equipped and integrated biotechnology research programme.

According to Richard Getzinger, head of international programmes at the AAAS, products from the programme already yield exports from Cuba worth \$125 million a year. The island also has internationally supported research efforts in ecology and marine biology.

But more traditional, university-based scientific disciplines, such as physics and chemistry, have collapsed since losing support from the former Soviet Union. "The crisis in physical sciences in Cuba is extreme," says Irving Lerch, head of international affairs at the American Physical Society.

Under its economic embargo of Cuba, the United States does not allow its own citizens to spend money on travel to Cuba, and does not allow Cubans employed by the



government — as most scientists are — to visit the United States. In theory, scientific researchers are exempt from both regulations, but many encounter problems when applying for permission to travel.

Luis Montero, for example, a chemistry professor at the University of Havana, has applied for an entry visa to the United States almost every year since 1990 to attend the same quantum chemistry meeting at Sanibel Island, Florida. His applications were granted in 1993 and 1996, but refused in 1990, 1991, 1994 and 1997.

In another case, OFAC recently turned down a request from Paul Walters, president of the American Chemical Society (ACS), to visit Cuba on a fact-finding mission. Walters had been invited by the president of the Cuban chemical society.

Asked at the meeting about the rejection, Clara David, a senior licensing officer at

OFAC, said: "Maybe the application was too general in its approach." John Malin, head of the ACS international section, says that he and Walters are now reapplying.

Some scientists who have had problems are incensed that the regulations require officials at OFAC, who have no scientific qualifications, to assess the legitimacy of proposed research. "Their level of disingenuousness continues to amaze me," says Ross MacPhee, a palaeontologist at the American Museum of Natural History in New York.

He says that several colleagues have had permission to go to Cuba denied on the grounds that they "can't just go there and look around". But just looking around, he points out, "is pretty much what a palaeontologist does!"

Michael Ranneberger, coordinator for Cuban affairs at the US State Department, says Cuba's human rights record is the reason for the embargo and the regulations. Most requests for travel in both directions are granted, he says, and "a great number of scientific exchanges have taken place".

Ranneberger says more than 80 per cent of Cuban researchers applying for visas to travel to the United States last year were successful, and that the 600 licences granted to US researchers to travel to Cuba last year constitute a majority of applications. He could not say how many US researchers were refused permission to travel.

The AAAS's human rights office is attempting to extract this information from the government using the Freedom of Information Act. Its report calls for an end to the licensing requirement, adding that, if it must continue, OFAC should ask scientific societies for help in assessing applications.

Kate Martin, a civil liberties lawyer, thinks researchers denied permission to pay for travel to Cuba could sue the government for breach of the First Amendment to the US Constitution, which guarantees freedom of speech. Because they need to travel in order to exchange ideas, "scientists have a stronger First Amendment right to travel than the rest of us," she says.

But some US researchers who have worked in Cuba say the regulations do not interfere with the sufficiently determined collaborator. Michael Smith, director of the Caribbean Biodiversity Program, says he held a briefing for OFAC officials early on, and has had no trouble getting permission to travel since. "We've had hundreds of licences [to visit Cuba] and only a handful of problems," he says. "We've also had hundreds of Cubans over here." Smith suggests that energy should be spent on proposing projects and making them work, rather than battering away at a government policy that is unlikely to change.

Colin Macilwain

Irish scientists face another budget blow

[MUNICH] Irish scientists were last week told that there is after all no money for new basic research projects in the 1998 budget of Forbairt, the government research agency in the Republic of Ireland. This comes despite a call for proposals last December and the completion of the grants review procedure.

It is the second time in five years that scientists have responded to a call for proposals only to find that there was no money to pay for successful applications (see *Nature* 364, 662; 1993).

Forbairt's basic research grant review committee was informally told last year that £6 million (US\$8 million) would be available. But it was given only enough cash to continue existing commitments. A spokesman for the Irish Office of Science and Technology, which oversees the agency, says the office will try to free cash from other sources to honour some of the projects the review committee approved for funding.

But David Fegan, a physicist who is a member of the grants review committee, says basic research seems to have lost its permanent home in a proposed government reorganization. The government, which came to power last autumn, wants to merge Forbairt with the Industrial Training Authority and the Irish Export Board into a new agency called Enterprise Ireland.

The new body will be responsible for the development of industry. Basic research would fit uneasily into its remit, but the government has not indicated where it would like basic research to be handled. One possibility is that it will return to the ministry of education. The ministry was last year given £5 million for research, yet could find no mechanism for allocating it. Researchers speculate that the Office of Science and Technology is relying on this money being made available for Forbairt's basic research programme.

Alison Abbott

British scientists in 'last chance' appeal

[LONDON] Last-minute efforts are being made to ensure that Britain's new Labour government includes a significant increase in science funding in its eagerly awaited comprehensive spending review.

The outcome of the review is likely to provide a public spending framework for at least the four years up to the next general election. Many in the research community therefore view it as a last chance to secure a commitment to enhanced support over this period.

Last week, the pressure group Save British Science launched a campaign, modelled on similar movements in the United States and Japan and intended to build public support for its case, to persuade the government to double its spending on research and development over the next ten years.

The move coincided with a report from the House of Commons Select Committee on Science and Technology, which calls for an urgent injection of at least £410 million (US\$684 million) for university laboratories and research equipment to stave off what it calls the "crisis" in university research.

John Battle, the junior minister for science, has already voiced his support for the efforts by Save British Science to secure more funding for research. Officials at the Department of Trade and Industry point out that

several recent funding decisions already point in this direction. For example, the annual budget announced last month by Gordon Brown, the Chancellor of the Exchequer, included the announcement that the government is to provide £20 million towards a new £50 million 'University Challenge' fund. The fund will provide seed capital to help universities to turn research results into commercial products.

Further contributions to this fund, whose creation was proposed by the committee headed by Lord Dearing that reported last year on the future of British universities (see *Nature* 388, 413; 1997), will come from charitable foundations. In particular, the Wellcome Trust and the Gatsby Trust have already promised support.

Last Friday, Battle also announced that the government has agreed to provide an extra £4.5 million to three programmes in space technology. These include £500,000 for a design study into a science satellite intended to demonstrate the new technologies needed for future collaborative exploration into deep space. They come on top of a £21.2 million package of investments in three programmes by the European Space Agency, announced last month and covering satellite multimedia links and Earth observation.

But the amounts of money involved in the decisions announced so far have been relatively small in terms of the government's overall expenditure on research and development, which totals about £2.6 billion a year. The key funding decisions will depend on the outcome of the spending review, set up shortly after the Labour party won the general election last May.

A key focus here will be the extent to which the government is prepared to provide additional funds to pay for the refurbishment of university laboratories, widely considered to be a top funding priority.

"There was an investment in infrastructure from the 1930s to the 1960s, but as we went into the 1970s, the investment dried up," Dave King, professor of chemical engineering at the University of Cambridge, said at the launch of the Save British Science campaign last week. "Many of us are working in laboratories that have not been refurbished since then, and indeed would not pass current health and safety standards." Save British Science argues that doubling investment in research over the next ten years "is not an ambitious objective, but a necessary one".

Addressing the decline in funding for infrastructure was also one of the main conclusions of the House of Commons select committee report published last week. The report is a response to the Dearing inquiry, which was set up by the previous, Conservative, government.

The Dearing committee recommended a £500 million fund providing low-interest loans to help universities to rebuild research infrastructure. The fund would be set up with money from both the state and the private sector. But the select committee rejected this suggestion, believing it to be unworkable. Members instead called for a cash injection from the government of between £410 million and £430 million.

The committee's report says it is the government's job to fund basic research infrastructure. It quotes witnesses from industry who argue that industry would be unwilling to contribute to a fund it could not control and from which it would receive no measurable benefit. The report says: "Until the financial state of universities improves... it would be absolute folly to encourage them to borrow money." Under-investment in research infrastructure is undermining researchers' ability to attract private-sector funding, the report adds.

Michael Clark, the committee chairman, says that major industry witnesses who came before the committee told how university scientists were knocking on their doors for access to advanced research equipment. "Ten years ago, it used to be the other way round," he says.

David Dickson & Ehsan Masood

NASA critics silenced as Mars loses face



Now you see it, now you don't: Mars Global Surveyor has shown the famous 'face' to be a rocky mesa.

[WASHINGTON] In youth the chin was strong, the gaze steady. Now, more than 20 years later, the Face on Mars does not appear to be its old self. The Mars Global Surveyor (MGS) snapped two new photos (centre and reversed at right) of the much-touted 'face' — actually a mesa of resistant rock in the Cydonia region of Mars — during a close pass of the planet last Sunday (5 April).

The new image was taken from a range of 444 km; each picture element has a resolution of 4.3 metres. Where some had imagined eyes, lips and a nose in the 1976 Viking picture (left), there now appear peaks, ridges and other features that show the face to be a natural geological formation.

The MGS is attempting several such targeted observations before resuming aerobraking operations in September to

lower its orbit. And for the US space agency NASA, taking the picture was a relatively simple way to silence conspiracy theorists who claimed that it had been trying to cover up evidence of the former presence of an 'intelligent' being on the planet.

But targeting small objects with Surveyor's fixed-view camera is "technically very challenging", says Michael Ravine of Malin Space Science Systems in San Diego, California, whose camera took the image.

Last week's attempts to photograph the much smaller Viking landers and Mars Pathfinder were not as successful. The camera track missed the Pathfinder and Viking 1 altogether, and the Viking 2 image was overexposed. But the MGS team will have two more chances before the end of April to get it right.

Tony Reichardt

Peer review cuts power of Italy's 'barons'

[MILAN] A new system for distributing national research funds to Italian universities appears to be working. Its first round of grants has been highly selective — resulting in many researchers experiencing their first-ever grant rejection — and has successfully encouraged greater collaboration between universities.

As a result, the government has increased the budget of the board that distributes the funds by more than 50 per cent this year. Part of this new money has been transferred from the funds of the National Research Council (CNR), whose role as a university grant agency the government wants to eliminate (see below).

Last year, the government abolished 14 discipline-orientated committees, each directly elected by the academic community, which had been responsible for allocating national university grants. It replaced them with a board of five individuals, selected by the research minister Luigi Berlinguer (see *Nature* 387, 538; 1997).

Under the new system, individuals may not apply for new grants. Instead, applications can be made only to extend, through collaboration with other researchers, projects already being financed from other

sources, such as the European Commission, and must be submitted by universities.

Successful bids involving collaboration with other researchers within the same university receive an extra 40 per cent on top of their original funding, while those between universities receive an extra 60 per cent. In February, the board approved only one-quarter of the applications it had received; 95 per cent of the approved grants were for inter-university collaborations.

The new system, based on independent peer review, is entirely electronic. A database of referees, now being extended to include foreign referees, and available for inspection on the Internet, includes an application form and updated information about the number of applications received.

The two members of the board who share responsibility for all the natural sciences, from agriculture to particle physics (social sciences, humanities and law are handled by the other three members), say they were initially concerned that five was too small a number.

Despite publishing referees' names, the system has been accused by some of lack of transparency. This is because board members have occasionally had to call on unnamed experts to help to select referees.

But the board came to agree with Berlinguer that the new structure is the best way to ensure that the tradition of personal contacts is eliminated. The member responsible for biology and medicine is Jacopo Meldolesi, a cell biologist and director of DIBIT, the research institute of the San Raffaele hospital in Milan. Meldolesi says he was immediately aware of intense pressure to return to the old system, particularly from the powerful academic 'barons' whose applications were rejected. Others criticized the abandonment of a 'democratic' approach to selecting grant review committees.

But Meldolesi says this sort of democracy is not suitable for science. "After all, the first violin in the national orchestra is not chosen by voting; people vote for the most popular, not the most competent." He is aware of his unpopularity in university circles. Despite a strong international reputation as a scientist, he has never served on a grant committee in Italy, and has long been an outspoken critic of a system favouring 'friendships' over scientific excellence. Like other board members, he was chosen by the ministry because he was not part of any university clique.

Antonio Contestabile, professor of neurobiology at the University of Bologna, says one weakness of the new system is that the whole academic community is fighting for a single pot of money that had previously been clearly divided between disciplines.

"This gives enormous space for conflicts of interest, cross-refereeing and manipulation of the final results by the still flourishing academic mafia," he says. "It will take years to reach international standard because we do not have a culture of critical refereeing."

But Alessandro Finazzi Agró, a biochemist and rector of Tor Vergata University in Rome, sees three positive effects of the system. First, approved projects received an average of 80 per cent of the money requested, compared to around 10 per cent under the old system. Second, because of this level of funding, the board will be able to evaluate the results of the grants. And third, he says, universities have been more careful about which projects they finance with their own funds, in order to attract as much extra money as possible from the ministry.

"Of course, in the first round of financing, big slices of Italian research were left out," says Agró. "But now there is some experience with the new system they will have more chance in the next round."

Carlo Calandra, director of the National Institute of Material Sciences, and chairman of the new board, says that the power of the 'academic mafia' will be further weakened by the rule that all applications must be submitted in English as well as Italian, so that foreign referees can be used.

Alison Abbott

Grants lure young researchers back home

[MILAN] One of Italy's largest research charities, the Italian Association for Cancer Research (AIRC), has become the first funding body to offer start-up grants to allow scientists who have worked abroad to return to Italy to take up a research career.

The grants cover salary costs for up to five years, and an additional sum of about IL100 million (US\$50,000) a year for direct research costs. AIRC's director, Giuseppe Della Porta, says the start-up grants are essential to stop Italy from losing its best young researchers.

One of the new grant-holders, Brambilla Riccardo, 35, a molecular biologist working at DIBIT, the research department of the San Raffaele hospital in Milan, says it would have been difficult for him to come back to Italy without the grant.

"Recruitment policy in Italy, which depends heavily

on personal contacts, strongly selects against researchers who go abroad," says Riccardo, who previously worked at the European Molecular Biology Laboratory in Heidelberg, Germany.

In addition to its normal investigator grants, the AIRC is offering coordinated grants to consortia of up to six different research groups in defined priority areas. In a bid to bypass the insularity of the Italian academic system, it has also changed its grant selection method.

All information is now provided in English, and applications must be submitted in English. The association's 12 study sections make a preliminary selection of the applications, which are then sent to referees, at least one of whom must be foreign.

Introducing similar reforms at Italy's National Research Council (CNR) has

proved more difficult. The government wants to break up the CNR's discipline-orientated committees (as it did the university committees; see above), which had previously allocated research grants, because of their apparent unwillingness to concentrate funding on the best research projects.

It wants the committees to be absorbed into a government-level 'scientific parliament', with purely advisory powers, and to strip the CNR of its responsibilities as a grants agency, partly because it wants a single grant allocation system.

But the CNR has resisted these changes, delaying their implementation.

A new proposal, which would also explain how the scientific parliament would work in practice, is expected to come from the government within the next couple of months.

A. A.

British BSE reckoning tells a dismal tale

Declan Butler

Evidence heard during the first four weeks of the public inquiry into the British government's handling of the BSE affair points to a catalogue of errors enacted against a background of spending cuts and deregulation.

[PARIS] Incompetence, excessive secrecy, and a failure to set up scientific advisory systems sufficient to meet the scale of the problem characterized the British government's initial response to the threat that bovine spongiform encephalopathy (BSE) might pass to humans.

This is the blunt picture that has been painted four weeks into the long-awaited inquiry on the circumstances that allowed the BSE epidemic to propagate throughout the UK cattle herd, culminating in the risk of an epidemic of a new variant of Creutzfeldt-Jakob disease (CJD) in people (see *Nature* 383, 658; 1996).

As evidence of the litany of shortcomings mounts with each day of the inquiry — ordered by the United Kingdom's new Labour government — it is becoming increasingly clear that the culture of deregulation and cost-cutting in both public services and science which dominated much of the 1980s also played a large part in determining events (see opposite).

The inquiry has also heard that a false sense of security resulted from the assumption that BSE was unlikely to pass to humans, given that scrapie has not been passed on despite being endemic in sheep populations for centuries (see *Nature* 384, 8; 1996).

The inquiry, which runs until the end of next year, is being carried out by a committee chaired by Nicholas Phillips, a judge in the UK

Court of Appeal. The other members are Malcolm Ferguson-Smith, professor of pathology at the University of Cambridge, and June Bridgeman, a former deputy chairwoman of the Equal Opportunities Commission.

So far, the £2 million inquiry has attracted unanimously enthusiastic praise from observers who say their first impressions are that it is "serious and thorough".

At the opening of each day's hearings, Paul Walker, the committee's legal counsel, tells witnesses that no-one is being criticized at this stage, which is aimed at "seeking only to establish and review the course of events". This, he warns, will come in the second phase of the inquiry, scheduled to begin in February 1999.

Observers predict that the inquiry is laying the factual groundwork against which later statements by politicians can be judged. The first round of hearings, which ended last Friday, has heard testimony mainly from scientists, including some who advised the government and others who claim to have been ignored.

In further rounds scheduled to run throughout the summer and autumn, ministers and their civil servants, as well as representatives from the meat and animal feed industries, will give their sides of the story.

More than 500 submissions of evidence have already been received, and the inquiry is expected to hear some 400 individuals. Faced with the complexity of the case, Phillips,

widely respected for his handling of the recent fraud trial concerning the late newspaper magnate, Robert Maxwell, has already extracted a six-month prolongation of the inquiry from Tony Blair, the prime minister.

Phillips' personal performance so far also seems to have allayed concerns about the inquiry's lack of powers to subpoena witnesses or documents. He has said that he will request such additional powers if he feels he needs them.

BSE was first officially identified in November 1986 by Colin Whitaker, a veterinarian. The case he identified was described by Gerald Wells, a neuropathologist at the government Central Veterinary Laboratory, Weybridge, in the first official report of the disease published by *The Veterinary Record*, in October 1987. But Wells admitted to the inquiry that this report was predated by two cases going back to 1985, which at the time were dismissed as sporadic events.

Wells claimed that faster action could not have been taken without waiting for more confirmed cases or drastically increasing surveillance. However, there was a nine-month delay in sending brain samples from the first case to the Ministry of Agriculture, Fisheries and Food (MAFF), with samples both being mixed up and sent to the wrong place.

The government's efforts to seek outside scientific advice on BSE began almost two years later with the creation in April 1988 of an ad hoc working party, chaired by Richard Southwood, professor of zoology and provice-chancellor of the University of Oxford. The working party's recommendation that a permanent expert group be set up led to the creation in February 1989 of the Tyrrell committee. This committee later became the Spongiform Encephalopathy Advisory Committee (SEAC), with David Tyrrell, then director of the UK Medical Research Council's Common Cold Unit, continuing as chairman until 1995, when John Pattison, dean of University College London Medical School, took over.

Southwood told the inquiry that his committee was "horrified" to learn at its first meeting in June 1988 that BSE-infected cattle were entering the food chain; a statement from a MAFF scientist that cattle heads were being removed using a chainsaw apparently did little to diminish their concern.

The committee considered it "essential" that measures be put in place to block the risk that "any part of an animal suspected of having BSE" ended up on consumers' plates, Southwood said. It also called on the government to launch a comprehensive research effort, and to make permanent a temporary six-month ban on the feeding of ruminant proteins to cattle, introduced in July 1988.

Indeed, the twin pillars of the government's strategy for containing BSE were the ban on potentially contaminated feed (see *Nature* 381, 544; 1996) and the November



Sitting in judgement: Nicholas Phillips (centre), June Bridgeman and Malcolm Ferguson-Smith.

PA NEWS

1989 abattoir ban on specified bovine offals. The feed ban should have cut off the main source of new BSE cases, and even if new cases arose, the offal ban should have stopped humans from eating infected material.

Even the government now acknowledges that neither ban was implemented properly. What Tyrrell claimed at the inquiry, however, was that, at the time, the government lulled his committee into a false sense of security by giving it an overly reassuring picture about the effectiveness of the measures that had been taken. "The assumption was that the offal ban was now in place," he argued.

Tyrrell thus defended the conclusion of an emergency meeting of his committee in May 1990 that: "The view was that the present risk, which could not be said to be zero, was not greater than the risks of everyday life, and thus beef could be said to be 'safe'." The meeting had been held to help Sir Donald Acheson, then the government's chief medical officer, to prepare a statement for a press conference to respond to growing public concern over the risks of BSE.

According to Tyrrell, SEAC became aware only in November 1995, six years after the specified bovine offals ban came into force, for example, that spinal cord was being left on carcasses. The minutes of the meeting recall that one member of SEAC was "appalled at this information". Similarly, the failure of the feed ban went unnoticed by government scientists until 1993, Tyrrell claimed, when they "began to be increasingly worried that cases were continuing to occur in animals born well after the ban should have been in full operation". In fact, almost half of the new cases occurring then were in animals born after the ban came into force.

"We did not have the expertise to follow through scientific conclusions which had practical implications," Tyrrell admitted to the inquiry. "Our view was that we knew little of the details of animal husbandry and modern abattoir practice, nor did we know about the regulations and the control and management systems in place."

His admission concurs with concerns already voiced in 1991 by Fred Brown, a researcher at the Plum Island Animal Disease Center in New York, and a member of SEAC. Brown had then advised the government that a more substantial coordinating body than SEAC was needed. In a letter the same year to Keith Meldrum, the then UK chief veterinary officer, Brown warned in particular: "I think you are expecting too much if you think a group of people meeting every two months or so can coordinate the work on BSE."

Brown also complained to government ministries about "the lack of coordination of research on the disease and its causative agent in the UK", and asserted that SEAC received "no information regarding the research work being conducted on such important topics as diagnosis and the nature of the agent". Tyrrell

Political initiatives contributed to crisis

[PARIS] Enthusiasm for public spending cuts and deregulation under Margaret Thatcher's leadership seems to have contributed directly to the BSE crisis, evidence heard by the public inquiry into the handling of the crisis has confirmed. This conclusion backs up a long-held suspicion (see *Nature* 384, 9; 1996).

Richard Southwood, head of an influential working party set up by the UK government in 1988 to recommend ways of handling the BSE outbreak, told the inquiry, for example, that Derek Andrews, then permanent secretary at the Ministry of Agriculture, Fisheries and Food (MAFF), had told him he hoped the committee's proposals "would not lead to an increase in public expenditure".

Southwood says the request did not influence the committee's work. But he added that it gave an idea of the political climate at the time. He says: "I'm sure it influenced [the government's handling of the BSE crisis]."

Similarly, David Tyrrell, chairman of the UK government's Spongiform Encephalopathy Advisory Committee (SEAC) until 1995, pointed out to the inquiry that general cutbacks in the veterinary service resulted in a neglect of the BSE problem as a whole and, in particular, that the lack of inspectors hindered enforcement of the feed and specified bovine offal bans.



Anderson: criticized a 'culture of secrecy' at MAFF.

Government cuts in science also curtailed research on BSE, argued Tyrrell, pointing out that MAFF's Central Veterinary Laboratory in Weybridge was at the time under intense budgetary pressure. And, in 1988, a visiting group had recommended that scrapie research at the Neuropathogenesis Unit in Edinburgh – itself faced with closure – be discontinued, he said.

Tyrrell added that the director of the UK Institute of Animal Health at Compton had also rejected a recommendation that it stop scrapie research, and that fortunately he decided to start BSE research programmes without waiting for earmarked funds, by transferring money from pig research.

Fred Brown, another member of SEAC, complains that MAFF concentrated BSE research at its Central Veterinary Laboratory, which had little previous experience of transmissible spongiform encephalopathies, and "tended to ignore" the more experienced Neuropathogenesis Unit. Roy Anderson, professor of zoology at the University of Oxford and a prominent expert on the epidemiology of

BSE (see *Nature* 383, 209; 1996), told the inquiry that scientific understanding of BSE had been delayed by the reluctance of MAFF to provide outside researchers with access to data – a tendency attributed to a 'culture of secrecy' at MAFF, and its failure to dedicate sufficient staff to analysing and distributing data (see *Nature* 383, 467; 1996).

The BSE crisis emerged at a time when the Conservative government was also keen to deregulate industry. Implicit criticism of this policy came from George Lamming, emeritus professor of biological studies at the University of Nottingham and chair of an expert working group on animal feedstuffs set up in 1991, who told how the government rejected his group's recommendation in 1992 that an independent committee be created to ensure that the feed industry was complying with the ban on feeding animal protein to cattle. Lamming told the inquiry that he was "extremely disappointed" with the government's reaction.

In an internal memo dated 28 July 1993, Brian Dickinson, then head of MAFF's Food Safety Group, wrote that such a group would "add to the pressures for regulation when we are trying to go the other way", and recommended lobbying the Department of Health, adding that "DH officials have been strongly in favour of the setting up of the committee".

D.B.

conceded: "Perhaps I should have been more receptive to [Brown's] idea."

Brown told the inquiry: "I felt that the problem was so big that it needed a coordinator to take hold of the whole thing. This was not accepted." Implicitly criticising Tyrrell's leadership, Brown also wrote that there was an "urgent need for the appointment of a coordinator who really knows the field".

Stephen Dealler, a scientist who repeatedly tried to alert the public to the potential risks of BSE, alleged that SEAC ignored his advice that many more infected cattle were going into the human food chain than was officially

recognized, and his doubts about the sensitivity and appropriateness of the mice assays used to evaluate infectivity in tissues.

Dealler complains that the setting of the BSE agenda was excessively concentrated in the hands of a few individuals within SEAC and the Central Veterinary Laboratory (see *Nature* 384, 201; 1996). He adds that the lack of public health experts on SEAC was belatedly corrected, for example, when John Patison as chairman recruited new members with expertise in this area. □

● The proceedings of the inquiry are accessible on: <http://www.bse.org.uk>

Peña quits job as US energy secretary after just 15 months



[WASHINGTON] Federico Peña (left), the US Secretary of Energy, announced this week that he will resign on 30 June after spending only 15 months at the helm of the department, which sponsors most US physics research.

A former mayor of Denver, Colorado, and Secretary of Transportation in the first administration of President Bill Clinton, Peña says he is leaving to spend more time with his family. He will quit politics and return to the private sector. He cites among his main achievements "addressing the management crisis at the Brookhaven National Laboratory and recruiting a solid team of managers at the department".

Peña's sacking of Brookhaven's contractor irritated some scientists (see *Nature* 387, 114; 1997). But the community applauded his appointment of Ernest Moniz, formerly chair of physics at the Massachusetts Institute of Technology, as under-secretary of energy with responsibility for science and technology.

A short tenure had been anticipated ever since Peña was unexpectedly given the job ahead of Elizabeth Moler, who became his deputy. Peña said nothing to dispel rumours that Moler is in line to succeed him.

Spain boosts the number of research jobs at last

[MUNICH] The Spanish government, after months of delay, has announced 65 new posts for junior researchers in the institutes of its national research council. A further 48 posts for senior researchers will also be provided through internal promotion.

On top of these increases, 55 additional positions for junior researchers are to be made available, but it is not yet clear if these will be permanent and who will be entitled to apply for them.

The promised increase in staffing comes as a welcome break for the hundreds of Spanish postdoctoral staff who trained abroad but returned home to broken promises of an expansion in research positions.

UK inquiry into genetic engineering of crops

[LONDON] The UK's Nuffield Council on Bioethics has launched an inquiry into the social, ethical and regulatory issues raised by the genetic modification of crops. A nine-

member working party is to be chaired by Alan Ryan, warden of New College, Oxford. Its members include Derek Burke, former vice-chancellor of the University of East Anglia and a former government adviser on genetically modified foods, Julie Hill of the Green Alliance, and Derek Osborn, who recently retired as permanent secretary at the Department of the Environment.

The working party will consider the safety and environmental impact of genetically modified crops, as well as implications for developing countries and intellectual property rights. Its final report is expected early next year.

Bill promises tax windfall for biomedical agency

[WASHINGTON] Americans would be able to direct their income tax overpayments to biomedical research at the National Institutes of Health (NIH) under a bill introduced by Representative Michael Bilirakis (Republican, Florida). Bilirakis chairs the health and environment subcommittee of the House of Representatives commerce committee.

Donations would be tax-deductible, and would add to NIH funding routinely appropriated by Congress. The bill is the latest in a flurry of proposed laws — mostly related to a proposed \$368 billion settlement with the tobacco industry — that aim to direct extra money to the agency.

'Red list' names 33,000 plants under threat

[LONDON] More than 10 per cent of the world's known plants are under threat of extinction, according to an inventory of threatened plants published yesterday (8 April) by the International Union for the Conservation of Nature. The 'red list' of threatened plants contains 33,000 species of vascular plants — ferns, gymnosperms and flowering plants.

The list has been compiled by the World Conservation Monitoring Centre in Cambridge. It is the result of a 15-year collaboration of botanical institutions and conservation organizations from around the world. The list will be put on the Internet (<http://www.wcmc.org.uk>), and will be used to set priorities in biodiversity conservation programmes.

Call for study of Europe's marine biodiversity

[PARIS] A major European effort is needed to describe and characterize marine biodiversity, and to quantify its economic importance. That is one of the recommendations of the European Science Action Plan on Marine Biodiversity, which is

to be released by the European Marine and Polar Science Boards, a body set up under the auspices of the European Science Foundation (ESF) and representing most of Europe's marine research organizations (see *Nature* 377, 469; 1995 & 392, 323; 1998).

The report claims that the understanding of marine biodiversity lags far behind that of terrestrial biodiversity. This means that the science needed to underpin conservation and sustainable use of marine resources is badly lacking.

The action plan will be submitted to the ESF and the European Commission. One member of the panel that prepared the report says it is hoped that it will stimulate new research programmes, especially under the commission's fifth Framework programme which starts in 1999.

US companies 'passed rocket secrets to China'

[LONDON] Two US companies that helped China with the launch of a satellite-bearing rocket are to be investigated by a US federal grand jury for the possible transfer of unauthorized technologies. The companies, Loral Space and Communications and Hughes Electronics Corporation, helped China to review the circumstances of the failure of a rocket carrying a Loral-built satellite in 1996.

US officials believe that the companies may have helped the Chinese to improve their rocket-guidance systems. This technology is known to be similar to the technology used to guide ballistic missiles. But, in a potential twist to the case, it has emerged that President Bill Clinton authorized Loral to launch a satellite aboard another Chinese rocket during an official investigation of the company's role in advising the Chinese on the failure of their earlier launch.

Japan puts blame for food poisoning on US

[TOKYO] Japan's Ministry of Health and Welfare announced last week that food poisoning outbreaks caused by the O157 strain of the *Escherichia coli* bacterium in July 1996 (see *Nature* 382, 567; 1996) were caused by radish sprout seeds imported from the United States. According to the ministry, genetic material unique to the bacterium was found in imported seeds.

Although the bacterium itself has not been detected, officials believe the evidence is strong enough to suggest that radish sprouts are the cause of food poisoning. But the US embassy and Japan's Ministry of Agriculture, Fisheries and Forestry remain unconvinced. The embassy contends that genetic material detected in the seeds could be from a different strain of *E. coli* that does not cause illnesses.

When rhetoric hits reality in debate on bioprospecting

The growing interest of pharmaceutical and agri-business companies in genes from natural products is generating a complex set of conflicts with Third World nations, where most of the world's genetic diversity is to be found.

[ST LOUIS, MISSOURI] By a twist of fate, the world's biological resources are distributed in approximately inverse proportion to its material wealth. The United Kingdom — typically for its size and latitude — has around 1,800 plant species. Peru probably has 18,000.

At the start of this decade, the economically poor nations of the tropics began to anticipate some payback from their biological riches. In 1991 the drug manufacturer Merck struck a pioneering, \$1 million deal with the Costa Rican government to exploit the biodiversity of the tiny central American nation's national parks.

Other drug companies, aware in particular of the success of the anti-cancer drug Taxol, derived from the Pacific yew tree, seemed poised to follow suit. At the 1992 Earth Summit in Rio de Janeiro, 140 nations signed up to a Convention on Biological Diversity, endorsing the concept of nations holding property rights to their indigenous species (see page 537).

The developing countries began to prepare for a gold rush of prospecting scientists from the United States and Europe. Their environmental ministers addressed the issue and made uncompromising public declarations of their readiness to strike a hard bargain — did everything, in fact, short of opening bars and brothels for the anticipated flood of bioprospectors.

But so far at least, the rush has not materialized. After all the hype, according to two dozen experts interviewed for this article, there is not much more bioprospecting activity going on now than there was ten years ago, although many in industry predict that interest is likely to accelerate, particularly in the search for new genes.

One reason for the current slow-down is the growing reliance of pharmaceutical companies on combinatorial chemistry, as opposed to natural products, as their favoured source for biologically active molecules that they can test as drugs.

Although most of what sits on the shelves of today's pharmacy was derived from natural products, the research arms of most drug

companies now see the systematic testing of synthetic molecules as the most promising route to tomorrow's therapies.

A commercial case for biodiversity conservation can be made on the basis of uses for natural products other than drug design. Better food supplements, and the genetic engineering of crops using genes from exotic species, are only two examples of multi-billion dollar businesses which could yet be built on the back of a fuller understanding of the world's plants, animals and microorganisms.

Where past bioprospecting activity has concentrated on the collection and identification of natural chemicals which organisms may use to protect themselves against predators or disease, the emphasis in future will be on the collection of genetic information from exotic species, for possible application in both genetic medicine and genetic engineering of crops. But both fields of activity are currently at an early stage of development, and no-one wants to make major investments in gathering and sequencing wild genes that they don't yet know how to use.

Great expectations

So action on these fronts will take decades, rather than years, to unfold — leaving developing countries well short of the expectations they held a few years ago. "There is this tremendous expectation on the part of the developing countries," says Stephen Blackmore of the Museum of Natural History in London, who argues that the alleged value of biodiversity for drug development was "hyped up" by conservationists. "It was a poorly developed argument for biodiversity conservation."

Western scientists involved in the collection and screening of

biological samples say that their work is harder to do — and far harder to find funding for — than many people realize. "From our point of view, it is developing a lot more slowly than we expected," says Robert Magill, head of research at the Missouri Botanical Garden, a major centre for botanical research. "It is not the windfall that we thought it might be."

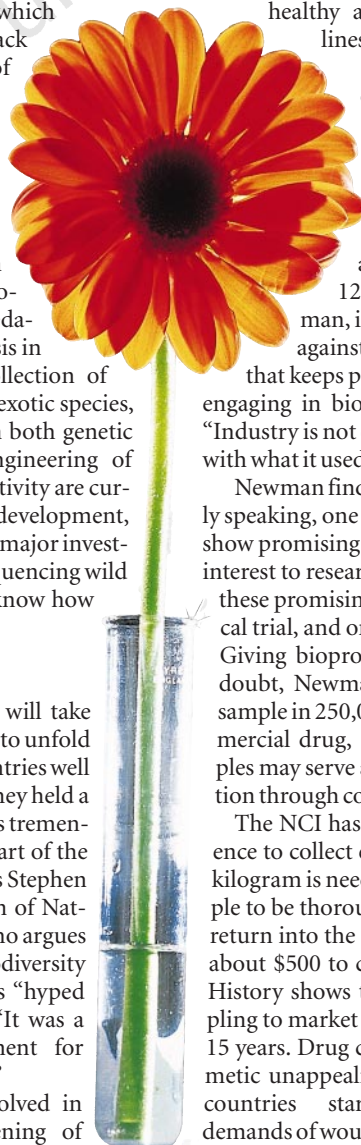
It is already possible to sequence genetic material from wild microbes and plants, with a view, for example, to the genetic engineering of crops. But the core of today's bioprospecting activities is comparable to what it has always been. Plants and other organisms are collected, ground down, dissolved, concentrated and tested against a target.

Thirty years ago, the US National Cancer Institute (NCI) would inject the resultant extract into cancer-ridden mice, and watch for the result — an immensely costly and time-consuming process. More recently, the concentrate has been tested in vitro against healthy and cancerous human cell lines.

David Newman is a senior chemist working at the natural products branch of the NCI's pharmaceutical development programme, which has collected about 70,000 samples of plants and microbes over the past 12 years. According to Newman, it is the extremely small odds against finding anything useful that keeps private drug companies from engaging in bioprospecting on their own. "Industry is not doing that much, compared with what it used to do," he says.

Newman finds this unsurprising. Roughly speaking, one sample in ten thousand will show promising activity in the area that is of interest to researchers, he says. One in ten of these promising samples might go to clinical trial, and one in ten of those to market. Giving bioprospecting the benefit of the doubt, Newman estimates that only one sample in 250,000 will directly yield a commercial drug, although many more samples may serve as useful leads for modification through combinatorial chemistry.

The NCI has learned from bitter experience to collect enough material — about a kilogram is needed — to allow a given sample to be thoroughly investigated without a return into the field, and each sample costs about \$500 to collect, transport and store. History shows that the process from sampling to market can take anything from 8 to 15 years. Drug companies found this arithmetic unappealing even before developing countries started making aggressive demands of would-be bioprospectors.



JOHN MILLER

India seeks tighter controls on germplasm

[NEW DELHI] *Lathyrus sativus* is a hardy pulse crop that is immune to droughts, needs no fertilizer and is 25 per cent protein. But there is a drawback: regular consumption can cause paralysis, as it contains a neurotoxin.

Scientists at the Indian Agricultural Research Institute (IARI) in New Delhi have now bred a toxin-free variety that could wipe out protein malnutrition in poor countries. But when Australian researchers recently asked for a few seeds to check if they were really toxin-free, they were given only a few grams of powdered — not whole — seeds.

The Australians were also told that if they wanted to grow the crop, they must negotiate a deal. "We have become wise," says Suresh Sinha, former director of IARI. Sinha complains that in the 1980s, Australia took away chickpea strains without payment, grew them up at home, and then exported them "not only back to us but to the Middle East".

Sinha is one of those who believe that India, which had been treating germplasm as a common heritage and freely allowing outsiders to collect, needs to protect whatever germplasm has not already been taken. "If you want the grain amaranthus that once grew wild in the Himalayas, you now have to go to the University of California at Davis," says Hari Krishan Jain, another ex-director of IARI and a former consultant to the Food and Agricultural Organization.

According to Jain, the only Indian germplasm not sitting in US gene banks is "what is available in our forests". And even this gap is being filled through joint projects, such as a \$300,000 study on the management of biological diversity funded by the US Department of Agriculture at the Wildlife Institute of India in Dehra Dun.

In a country where political sensitivities still run deep over 'exploitation' by multinational corporations — much of it focused on such corporations' efforts to claim patent rights for extracts from plants of Indian origin, such as the high-profile dispute over the 'neem' tree (see *Nature* 377, 95; 1995) — many scientists nevertheless agree that the exchange of germplasm between countries is necessary to develop new varieties and improve food production.

"The United States used to tell us that germplasm is common heritage," says Jain. "But once biotechnology became interested in the 1980s, it dropped the heritage philosophy and called for patent protection."

India's current efforts to conserve its agro-biodiversity centre on the \$20 million national gene bank commissioned last year, with its network of ten regional stations and 30 active collection centres spread around



Ancient or modern? An itinerant ayurvedic vendor in Kerala State, India.

the country. Built with US funds, "it is the third biggest seed bank in the world with a capacity to store one million samples for several years", says P. L. Gautam, director of the National Bureau of Plant Genetic Resources (NBPGR), the nodal agency for plant exploration, conservation, germplasm import and export.

At present the bank has 167,236 base collections, and Gautam says he is trying to get back all its original germplasm from international gene banks. So far, only 9,000 samples have been returned at NBPGR's request. And none has yet been requested from the bank holding the largest collections from India — the US bank at Fort Collins in Colorado. But Rajendra Singh Paroda, director general of the Indian Council of Agricultural Research (ICAR), says he does not foresee a major problem in getting the germplasm back.

Paroda says the conservation of agrobiodiversity is at the top of ICAR's agenda, and that the export of germplasm will be regulated by legislation being drafted in accordance with the UN Convention on Biodiversity, which India ratified in 1994. The legislation will prohibit open access to the Indian gene bank by US seed companies — access that was allowed under a 1988 agreement with the United States.

The draft biodiversity legislation, which is designed to combat 'biopiracy' and has still to be approved by parliament, proposes to regulate access to the country's biological resources by foreign researchers and pharmaceutical companies. Indian scientists who transfer commercially exploitable research results to outsiders without official approval would be punished. **K. S. Jayaraman**

The NCI requires anyone who exploits its repository to involve the country of origin in the development of any derivative products, and to pay royalties that increase with the scale of exploitation. But in some places, Newman says, this is no longer enough. "We've stopped collecting in some countries because they wanted things that we just couldn't give," he says (see page 538).

In a bid to satisfy these new demands, three US bodies — the National Institutes of Health (NIH), the National Science Foundation and the US Agency for International Development (USAID) — established a programme in 1992 to build four-way partnerships between industry and science in both the United States and in the donor country.

Five of these International Co-operative Biodiversity Groups (ICBGs) have been established (although the USAID has withdrawn from the programme), each costing the US government about \$500,000 a year to run. "They have been very challenging to set up," says Josh Rosenthal, who runs the programme from NIH's Fogarty International Center (see page 539). "It is very novel terrain."

"The idea is that the groups should serve as models for how drug discovery could be done, while serving broader conservation goals," says Rosenthal. He adds that industrial corporations are ready to share costs with host countries and to pay royalties to them — but are not ready to make major, up-front investments to meet these conservation goals. "We'd like to see the private sector do it, but my sense is that that is not going to happen. Industry isn't interested in raising its budgets for natural products research."

Synthetic alternatives

Instead, industry is pursuing increasingly powerful techniques for developing drugs based on molecules that scientists can create for themselves in the laboratory, using new combinatorial chemistry techniques.

Until a few years ago, the *in vitro* screening of a given compound against a target was a time-consuming process that constrained the number of samples that the drug companies needed or wanted to test. The task was to select the most promising compounds for test. These were usually natural products.

Today, however, assays can be done 5,000 at a time in automated machines, and the sources of properly documented natural products can't keep up. Combinatorial chemists can use modern techniques to create arrays of thousands of slightly different molecules, ready to go into screening and, perhaps, feed the drug firms' insatiable appetite for a 'hit'.

At present, all these synthetic molecules are polymers. The understanding of structural biology doesn't allow for the creation of anything with such a novel structure as, say, Taxol, which consists of four intricately

entwined carbon rings. But even simple polymers have their uses, and combinatorial chemistry gives the developers complete knowledge and control over what they are working with.

Some supporters of combinatorial chemistry question the value of biodiversity as a source of new drugs. "I think it leaves [biodiversity] completely high and dry," says David Galas, formerly head of the Department of Energy's human genome sequencing efforts, and now president of Darwin Molecular, a biotechnology company based in Seattle.

"As we learn more about three-dimensional structures, it appears there is nothing special about natural products," he asserts. For Galas, the study of biodiversity is useful primarily because it provides insight into what evolution has produced. "The idea of

exploiting the rain forests to find wonderful drugs is, quite frankly, not credible," he says.

Galas further claims that romantic sentiment is blinding plant scientists to this reality. "Plant scientists have come to have such reverence for plants that you'll find a lot of reverence for natural products — but I think it is misplaced."

Most other observers, however, expect that a combination of natural and synthesised products will lead to future drugs. "Both paths have a very rich future," says Robert Horsch, manager of Monsanto's Agracetus Campus at Madison, Wisconsin. The ability to synthesize proteins is growing exponentially, he says, "but we can't even dream of making all possible combinations. Nature has been trying this experiment for two billion years".

Eric Fischer, head of the science, technology and medicine division of the Congressional Research Service in Washington, DC, and former director of the biology board at the United States' National Research Council, concurs. Fischer says our current understanding of structural biology is far too shallow to allow for the synthesis of the kinds of molecules that nature can produce. "Natural prospecting can get you whole new classes of materials that you couldn't even have imagined," he says.

The biological approach

At present, however, that fact isn't sufficient to push major drug companies to invest seriously in bioprospecting. To give that impetus, scientists advocate a more selective approach, based on a better understanding

Social equity versus private property: striking the right balance

[LONDON] Nowhere do the rhetoric and the reality facing bioprospecting come into sharper conflict than in attempts by developing countries to bridge their international commitments to two separate agreements. These agreements are the UN Convention on Biological Diversity, signed at the 1992 Earth Summit in Rio de Janeiro, and the Trade Related Aspects of Intellectual Property Rights (TRIPs) agreement of the World Trade Organization (WTO), which came into effect in 1995.

As far as the Biodiversity Convention is concerned, bioprospecting will move to centre stage next month, when representatives of more than 170 countries gather in Bratislava, Slovakia for the fourth 'conference of the parties'.

A key issue there will be how to reach a compromise, between the commitments to accessibility and equity enshrined in the convention and the pressures for private ownership and profit-based systems of reward represented by TRIPs.

Four of 42 articles relate to bioprospecting. They broadly require governments to outlaw bioprospecting without a host country's consent and for the results of research, and benefits arising from commercial use of genetic resources, to be shared "in a fair and equitable way", on mutually agreed terms. Beneficiaries are to include traditional communities, and innovations to traditional medicinal compounds need their "approval and involvement".

Signatory states are supposed to incorporate these articles in national law. About 80 developing countries are addressing the task. But so far progress has been slow. Calestous Juma, the convention's executive secretary, says this is mainly because countries lack the capacity to enforce regulations. He says that laws require



Seeds of confusion: the WTO and the biodiversity convention appear to be at odds over patent rights.

"reciprocal arrangements in countries importing biological resources for them to be fully effective".

But another reason for the delay is TRIPs. Not only does this agreement appear to conflict with the spirit of the convention, but it also has teeth: if WTO members do not sign up to TRIPs, they face trade sanctions.

TRIPs contains a detailed framework for intellectual property rights, which specifies that, although plants and animals as such do not necessarily have to be covered, microorganisms and "essentially biological processes for [their] production" must be.

There is no requirement on applicants to involve or consult with local communities or governments about patenting a compound based on a natural product from that country. Nor is there provision for sharing benefits or including the prior contributions of indigenous peoples to an innovation.

The Biodiversity Convention states clearly that legislation on intellectual property rights should "not run counter" to

the convention. Many developing countries believe TRIPs is in clear breach. But the WTO disagrees, arguing that there is no conflict between the two.

Admittedly, TRIPs acknowledges the right of countries to decide on the details of their own patent systems. But other WTO member states do not have to honour such systems, and can mount a challenge to them.

Unlike the biodiversity convention, TRIPs carries a timetable for compliance. Developed countries had to comply by 1996. The larger developing countries, such as India, China and those in Latin America, must harmonize their patents legislation by 2000. The least developed countries are given a further five years.

While countries such as Brazil and Argentina are rushing to get TRIPs onto their statutes, others — notably many in Africa — are therefore in no hurry.

In Africa, a declaration issued last month by the Organization of African Unity's (OAU's) task force on access to genetic resources argued that TRIPs should comply with the biodiversity convention, and not the other way round (see *Nature* 392, 423; 1998).

Johnson Ekpere, executive secretary of the OAU's Scientific and Technological Research Commission, describes the WTO's approach as "predatory". He claims it "runs counter" to the biodiversity convention, as well as the "aspirations of communities which are in the first place the innovators of biodiversity". The OAU is pushing member countries to adopt its own model legislation before the first review of TRIPs next year.

But David Downs, a senior attorney with the Centre for International Environmental Law in Washington, DC, says the review will focus on trying to reopen the exclusion of patents on patenting plants and animals — not bringing TRIPs into line with the biodiversity convention.

Ehsan Masood

ANDREW MCROBB

Brazil's scientists warn against 'nationalistic' restrictions

[SÃO PAULO] Brazilian scientists are worried that a law aimed at protecting the 'theft' of genetic resources by foreigners — particularly from the biodiversity-rich Amazon region — could become a major obstacle to future research programmes in the area.

The law, which is being debated in the Brazilian Congress, is intended to meet the country's commitments following its ratification of the biodiversity convention signed during the Earth Summit in 1992 in Rio de Janeiro.

As in India and Africa, the biodiversity issue has become a battleground for opposing political views. But Brazil's scientific community is in a stronger position to work in a genuine partnership with foreign researchers, and this is leading to a more pragmatic stance than that found in India and Africa.

The government of Brazilian president Fernando Henrique Cardoso is seeking relatively liberal legislation, which would provide scope for negotiation between institutions representing the domestic and foreign researchers involved in the discovery. Under this legislation, the institutions would be able to negotiate the patent rights to any genetic resources discovered that proved to have commercial value.

Left-wing opposition parties are fighting for a more nationalistic law to halt what they describe as 'genetic colonialism' from First World countries and their research institutions. Government officials fear that if this sentiment wins the day — which many feel is likely — Congress may create a genetic equivalent to the protectionist legislation on information technology, which was seen by many as having been an obstacle to the development of the Brazilian computer industry in the recent past.

The biodiversity bill has been under discussion since 1995. Initially proposed by Marina Silva, a senator from the Partido dos Trabalhadores (Workers Party) who represents the Amazonian state of Acre, it has already served as a basis for legislation passed by the Acre state Congress in July 1997.

This legislation is considered by many researchers to be almost xenophobic, as it forbids foreign researchers from entering the Amazonian forest without a formal agreement involving Brazilian researchers as partners. It was proposed by state deputy Edvaldo Magalhaes, from the Communist Party of Brazil, who warned that the country needed to defend itself against "a sort of new colonialism".

The political debate has been particularly stirred up by press stories about foreign



Endangered species? New rules may threaten joint projects with foreign researchers (above).

researchers in Amazonia who have sought patents abroad based on micro-organisms and genetic material whose existence they learned about from Amazonian Indians.

One British chemist, for example, was said to have sought to patent the substance rupuninine, obtained from the bibiri tree (*Octotea rodioei*) found in the frontier region between Brazil and Guiana and known to have contraceptive properties; and cumaniol, a poison made from cassava leaves which can stimulate the central nervous system. Both substances are used by the Wapishana Indians of Roraima.

A new intellectual property law, which still needs to be approved by the president, already includes an article protecting "traditional and ethnical knowledge". But scientists fear that if the genetic resources law is too restrictive — for example, by making it a criminal offence to take genetic material out of the country without official permission — it will in practice scare away foreign researchers who might otherwise come to work in Brazil.

"Just outlawing won't help. We won't even know what's being stolen," says Sergio Henrique Ferreira, a biomedical researcher at the University of São Paulo and president of the Brazilian Society for the Progress of Science (SBPC). "The major issue is the need to protect intellectual property."

Glaci Zancan, a biologist at the Federal

University of Paraná and a vice-president of SBPC, adds: "All we need is a simple clause: if something patentable arises from the research, then we'll negotiate over how the rights to it should be shared."

"The most important point is to ensure that the terms of joint projects are properly negotiated in advance," says Marcio de Miranda Santos, head of the Research and Development Department at Embrapa (Brazilian Agricultural Research Corporation). "There would be no point in having an excellent law but second-rate scientists."

Maria José Amstalden Sampaio, a researcher at Embrapa who also acts as an adviser on legislative affairs, says that, despite some minor improvements since 1995, the proposed law is still too complex and detailed, making compliance virtually impossible.

For example, she points out that the text of the law even includes model contracts covering access to genetic material. "This should be dealt with on a case-by-case basis," she says, rather than by a single form of contract.

"The law is not even flexible enough to distinguish between the different needs in agricultural or biomedical research."

Sampaio says that she has been following closely what other countries have been doing. "Take the Philippines law, which is so tightly written that it makes negotiations quite difficult." She has detected this same approach in other countries. "I like to joke with Colombians: OK, if you close your forest [to researchers], then we will open ours, and everybody will come here".

According to many Brazilian scientists, another threat to the future ability of Brazilian researchers to develop the country's genetic resources is recent cuts in the number of scholarships provided by the Ministry of Science and Technology.

Amazon research attracts most of the foreign scientists seeking to carry out research in Brazil. "Sometimes it is difficult to find enough Brazilian personnel at the same level to meet the demands," says Renate Stille, head of the Science and Technology Division at the Brazilian Ministry of Foreign Affairs.

Already under Brazilian federal law, any foreign scientist wanting to work in the country as part of what the law calls an 'expedition' must be accompanied by, and confer co-authorship to, Brazil researchers. Field activities performed by foreign researchers are considered to be scientific expeditions, says the Ministry of Science and Technology, and thus are covered by the regulation.

Ricardo Bonalume Neto

ANDREW MICROBB

of systematic biology — allowing more suitable plants and microbes to be chosen for sampling — and on the study of the use of natural products by indigenous peoples.

Peter Raven, director of the Missouri Botanical Garden and one of the most prominent plant scientists in the United States supports both approaches. “Most useful drugs come from molecules that people were already using,” says Raven, who returned last month from visiting India.

A formula for indigenous involvement

[LONDON] Sharp controversy over the ‘ownership’ of natural products in Africa (see page 540) does not seem to have dented the popularity of the International Cooperative Biodiversity Group (ICBG), an expanding network of bioprospecting projects sponsored by the US government through the National Science Foundation, and the National Institutes of Health (NIH).

ICBG projects aim to find plants with ingredients that could treat priority diseases in the United States. Four ICBG networks operate in Latin American countries. But the largest one is in Africa, taking in Nigeria and Cameroon.

Countries hosting the projects can expect financial rewards for local people, investment in research into priority diseases, a share in royalties from sales of drugs and strengthening of local institutions engaged in research and traditional medicine.

The scheme is popular. Thirty-four projects from 25 countries — mostly in Latin America and the Caribbean — entered in the last wave of applications. Even more have applied for the next tranche, out of which six will be chosen. Each project receives about US\$500,000 a year — a sum that the ICBG’s programme manager Josh Rosenthal acknowledges could be bettered.

The projects involve collecting samples from medicinal plants by, for example, negotiating access to traditional healers’ associations, herbal gardens and pharmacies. In return, the healers are paid a fee, and are advised on primary health care, offered help with setting up apprenticeship schemes in traditional healing and with testing the ingredients of their remedies.

As far as possible, local laboratories are used to analyse plants for potentially useful compounds, and for fractionation to isolate those compounds. Further analysis, and clinical trials on compounds with potential anti-viral or anti-cancer activity, is carried out in the United States by public- or private-sector laboratories.

If a compound were to lead to a successful drug, the bulk of royalties would be invested in the host country. In Africa, 20 per cent would go to the inventors, 50 per cent into a community development trust

“Thousands of materials have been catalogued in India and China. It is true that the large drug companies aren’t spending much time looking at them now — but they will.”

Although “one could imagine a partnership between systematic biology and the drug companies” to increase the efficiency of bioprospecting, he says that isn’t happening because “it is just not promising enough”.

Another area of significant potential is the prospecting of less-studied forms of life

fund run by local people and 30 per cent towards research into tropical diseases at the Walter Reed Army Institute.

The private sector’s degree of involvement provides the essential difference between ICBG projects in Africa and Latin America. Latin American projects involve private-sector companies upfront. This is not the case in Africa, where governments are more wary of foreign multinationals and where the issue of patenting is more sensitive (see page 540).

In Africa, where much thinking has gone into the design of ICBG projects, private-sector involvement is restricted to contract laboratories. “Governments are far more comfortable dealing with governments,” says Maurice Iwu, a key player in ICBG Africa and director of the Bioresources Development and Conservation Programme in Lagos.

Similarly, botanic gardens, given the controversial nature of their previous involvement in Africa, have not been invited to participate in the projects. “Basically, we have learnt from history,” says Iwu.

But some remain unconvinced by the ICBG. Tewolde Berhan Egziabher, for example, general manager of Ethiopia’s environmental protection agency, and a key figure in the draft OAU convention on access to genetic resources, describes the ICBG approach as “an improvement in garb, but not in substance”.

“In the past, research organizations from overseas would simply walk off with our plants. Some still do,” he says. “I would like to believe that there is goodwill, and that institutions from countries such as the United States are working in our interests. But history tells me something else. Which is why I have yet to be convinced.”

But Iwu says that in his experience, attitudes to conservation and development in the United States and in European countries are changing. Government agencies appear keen not to repeat past mistakes. He says they are prepared to listen more and dictate less. And he says it is up to Africa’s governments to rise to the challenge, and derive maximum benefit from this new climate of partnership.

Ehsan Masood

on earth, including insects and microbes. “People are becoming aware of vast categories of organisms that have never been looked at,” says Tom Eisner of Cornell University, New York.

This area is also proving controversial. Last year, for example, the US Park Service reached an agreement with Diversa of San Diego, California, to allow it to search for heat-resistant micro-organisms in the hot springs of the Yellowstone National Park. But this pioneering deal is causing trouble within the United States: environmental groups have charged that it is unlawful (see *Nature* 392, 117; 1998).

Perhaps most significant, however, is the vast potential that prospecting for genetic information is opening up. Where past emphasis has been on finding organic molecules that will perform some biochemical function, future bioprospectors will seek gene-sequence information for use in medicine and in agricultural biotechnology.

But for now, such applications are under-developed. According to Raven, we don’t yet know enough about the relationship between different genomes to use sequence information from exotic species. “Right now, it’s a question of finding genes to do what?” he says.

The organization most likely to answer that question is probably Monsanto, the former chemicals manufacturer based in St Louis, which has converted itself into a multi-billion dollar life sciences corporation with major interests in pharmaceuticals, agricultural products and food additives.

The view from industry

Bob Shapiro, Monsanto’s chairman since 1995, has sold Wall Street a new image for his corporation, based around such unfamiliar concepts as biodiversity and sustainable development, and watched its stock market value balloon from \$7 billion to \$31 billion since 1995. During that time, Monsanto has brought the first genetically modified crop strains into mainstream use, culminating ten years of research at its vast research village at Chesterfield, outside St Louis.

“We’ve had projects that involve getting genetic material from exotic locations,” says Shapiro. “But now there is so much out there on Internet databases that you can do a lot without leaving home. To really take advantage of the materials out there is a 50-year task. But it definitely will happen.”

Shapiro says he is “uncomfortable” with the concept of biodiversity as a resource for his corporation to exploit. Instead, he says, Monsanto is interested because “diversity gives you the best chance of being robust”. “It’s easy to get romantic about biodiversity conservation,” he says. “But no-one knows what the uses of the genes will be. It would be idiotic to say that they’ll be of no value.”

With the recent introduction of crops

that resist pests because their genome incorporates genes from *Bacillus thuringiensis* (Bt) — a naturally occurring organism — and others genetically engineered to tolerate Monsanto's Roundup weedkiller, the St Louis corporation has led a sometimes-sceptical world into a new age of genetically modified crops.

David Fischhoff, Monsanto's director of advanced genomics, expects systematic bioprospecting to play a larger role in future. "When we started developing resistant crops, the world had at its disposal a few hundred genes," he says. "For agricultural purposes, we had a handful of them — and out of that, Monsanto has started some very successful projects.

"We're now about to have some understanding of literally tens of thousands of genes," he adds. "Bioprospecting for genes

will become more important than bioprospecting for proteins and organisms has been in the past."

Need for dialogue

An equally broad array of opportunities could exist in the use of genes from natural products to modify foodstuffs to enhance human health, says Ganesh Kishore, co-president of Monsanto's nutrition branch. Kishore envisages the discovery of natural genes that can be added to foods such as cooking oil, and so reinstated in the food chain.

But the Indian-born scientist has a warning for his former compatriots: "When I harness a gene, the fruits of the work are for everybody — but the person who developed it was me," he asserts. Like others involved, Kishore is deeply concerned about the state of relations between the rich and poor nations

on this topic. He says the parties haven't struck up the right conversation because "there has been a polarization, and we've ended up arguing over who is in the wrong."

Kishore says that Monsanto will "put its best foot forward" to make arrangements with countries such as India. But the Missouri life sciences corporation knows that it can expect to encounter a great deal of suspicion as it seeks to extend its leadership position in agricultural biotechnology (see *Nature* 388, 817 & 389, 534; 1997), and Kishore's stance on property rights is unlikely to go down well in developing countries.

The gap between what the developed world wants from biodiversity, and what the developing world thinks it should retain for itself, seems to be widening. Tensions between the two sides seem unlikely to relax in the near future.

Colin Macilwain

Old scores surface as African states face new opportunities

[LONDON] Feelings about 'bioprospecting' run high in Africa. The opposition is based partly on memories of colonial times, when areas of the continent were used as a free source of plants by staff from colonial powers' botanic gardens. But there are signs that this historical antagonism is beginning to soften, and that a pragmatism based on self-interest is starting to emerge.

The antagonism is still present. At the end of last month, a task force from the Organization of African Unity (OAU) put the finishing touches to a draft model bill on "community rights and access to biological resources".

The draft, heavily influenced by Ethiopia and the OAU's science commission, seeks to penalize those engaged in bioprospecting without permission from governments. More controversially, it refuses to recognize patents on compounds based on natural products, unless the patent recognizes the 'ownership' and contribution of indigenous peoples (see *Nature* 392, 423; 1998).

The draft will be circulated to the OAU's 53 member states, to be discussed at a meeting in June. It is designed as a template for legislation in individual African states.

Its origins lie in a meeting of African ministers in 1993. This meeting decided to ban all bioprospecting by overseas organizations until Africa-wide legislation was put in place. But the intervening five years have seen a change in thinking, a new pragmatism, and a breaking of ranks.

Many individual African states say that while they support strong legislation in principle — as they still find it difficult to trust bioprospecting initiatives from former colonial rulers — they do not believe this should stop them negotiating deals from which they stand to benefit.

The states are also more relaxed about the



Plant power: growing *Ancistrocladus korupensis*, a potential anti-cancer drug, in Cameroon.

patents issue. They realize that the financial rewards from drug development are long-term and not guaranteed, particularly as it can take 10 years for a drug to come to market and only 3 per cent of drugs that undergo development stand a chance of commercial success. These countries are looking for other kinds of benefits.

Nigeria, Ghana, Tanzania, and Guinea are among those countries that have taken such a pragmatic stance.

Cameroon is hesitating. It has blocked at the last minute a proposed agreement to allow the US National Cancer Institute to study the forest vine *Ancistrocladus korupensis*, a promising anti-HIV agent, in August 1993. But the state has allowed universities to take part in another project sponsored by the US government.

Ethiopia, in sharp contrast, remains opposed to both bioprospecting and patenting. Ethiopia's stance is significant. It was once a colonial power in its own right, and its historical influence lingers in Africa.

Nigeria and Cameroon have become part of a bioprospecting network, the International Cooperative Biodiversity Groups (ICBG), which was set up and

sponsored by the US government. The network's African arm includes two US research institutions and 13 in Nigeria and Cameroon.

The ICBG aims to find plants from Africa's forests that could provide drugs to treat priority diseases in the United States such as AIDS, cancer, and disorders of the cardiovascular and central nervous systems. In return, the host country is promised financial remuneration, as well as a range of benefits such as a boost for local research capacity and spin-off benefits for traditional medicine.

The sponsors promise that patent applications will include the names of all possible contributors to the invention — including scientists, local communities and traditional healers.

But for Ethiopia and the OAU's scientific commission, these promises are not enough. According to Johnson Ekpere, executive secretary of the OAU's Scientific and Technological Research Commission, a bioprospecting arrangement must find an alternative to patenting.

Ekpere says he is sympathetic to the aims of the ICBG. But he believes another method has to be found to balance the desire to protect an invention with what he describes as a country's "right" to safeguard the sovereignty of its natural products, and to provide shared and free access to an organism.

But Maurice Iwu of ICBG Africa argues that the ability to patent is integral to the concept of bioprospecting. He says that no overseas partner will engage in bioprospecting unless the results of research can be protected in a patent. Iwu says the ICBG's patenting proposals are designed to "be of maximum benefit" to the host country.

Ehsan Masood

Numbers of lab animals questioned

Sir — The report on US laboratory animal populations¹ contains the surprising assertion that there is an increasing use of animals in research in the United States and that lack of laboratory animal space is threatening the existence of valuable disease models. The proposed remedy includes an infusion of money to expand laboratory animal space.

Such US data as are available indicate that laboratory animal use is, in general, declining. It may be declining as dramatically as it has in Great Britain (more than a 50% decline since 1975), the Netherlands (50% since 1978), Switzerland (75% since 1983) or Germany (35% since 1989). Although the US data are incomplete because mice and rats are not tallied, despite making up 90% or so of the laboratory animals used, the annual report of dog, cat, primate, hamster, guinea pig and rabbit use shows that use of these six species has declined by about 40% since 1976. A report on laboratory animal use by the Department of Defense found that rat use declined by 49% and mouse use by 28% from 1983 to 1991 (ref. 2).

Another study pointed out that, among 20 commercial laboratories providing such data in their annual reports, rat and mouse use had declined by 39% from 1986 to 1994. Rat and mouse use at the National Institutes

of Health (NIH) fluctuated between 1986 and 1993, but mouse use was highest in 1989 (at around 450,000) and then declined to 300,000 in 1991 before rising again to about 380,000 in 1993. Rat use declined slowly but steadily over the same period³. A 1966 report notes that in 1965 NIH used 670,000 mice and 163,000 rats⁴.

Although the available data also indicate that the use of mice (especially in the development of disease models using transgenic and knockout technology) has increased steadily over the past five to ten years, the claim that animal use in general is rising or that the lack of animal holding capacity has reached a crisis is not supported by either the data or by my personal experience of the space availability at four research institutions in the northeast United States from 1984 to 1997. Of course, if investigators now wish to set up facilities to maintain breeding colonies of new mouse models of human disease, then competition for animal space may well develop. Most American institutions are not set up to handle *in situ* breeding and colony maintenance because they usually purchase animals as needed from commercial suppliers.

The trends in biomedical research for the past 20 years have been away from animal use towards molecular and cellular

investigation. The excitement about knockout and transgenic techniques may result in a reversal of the worldwide downward trend in laboratory animal use, but such a reversal is likely to be temporary. Like Sir Peter Medawar in 1969, we look to a future where our growing knowledge of biology — obtained through both animal and increasingly non-animal research — will “provide us with the knowledge that will make it possible for us, one day, to dispense with the use of [animals] altogether”⁵.

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1. Wadman, M. *Nature* **391**, 623 (1998).
2. Weichbrod, R. (PhD Thesis, Walden University Institute of Advanced Studies, 1993).
3. Davis, C. *Animal Use Trends in the United States, 1986–1994* (WARDS, 8150 Leesburg Pike, Vienna, Virginia, 1996).
4. *The Care and Management of Laboratory Animals Used in Programs of the Department of Health, Education and Welfare* (Division of Operations Analysis, Office of the Comptroller, DHEW, 1966).
5. Medawar, P. B. *The Hope of Progress* (Methuen, London, 1972).

● The author's disagreement is with the findings of the US National Research Council, not with our report of those findings. — Editor, *Nature*.

Whither whaling?

Sir — A number of parties to the International Whaling Commission (IWC) met in Antigua on 3–5 February to consider a plan which, its supporters suggest, could end the existing stalemate between pro- and anti-whalers. Despite the moratorium on commercial whaling agreed in 1982, Japan still takes whales under the guise of scientific research, and Norway continues to hunt using a formal objection to the moratorium. Their combined annual take is more than 1,000 minke whales.

The whalers were invited to phase out their whaling and agree to an international trade ban. In exchange, they were offered IWC-endorsed quotas in their domestic waters. Although the rationale for such a compromise has been outlined elsewhere^{1,2}, many conservation groups remained concerned that the deal would end the moratorium, strongly signalling that commercial whaling was again internationally approved. But, although the issue remains on the agenda for the IWC meeting in May³, it seems that the whalers themselves have rejected the compromise. Arguments that coastal whaling

communities deserved quotas to alleviate hardship seem to have lost out to their desire for widespread whaling and trade, appearing to confirm that their motives are purely commercial.

The IWC Scientific Committee is making assessments of whale stocks that will lead to theoretical quotas for various species (even though such quotas have been unilaterally made a reality by Norway for North Atlantic whales). Scientific support for sustainable use also influences the Convention on International Trade in Endangered Species (CITES). At its last meeting, those keen to remove existing trade restrictions on minke whales were only narrowly defeated.

Commercial whaling, however, meets no pressing human need and — however good the modelling — subjects whale populations to unnecessary risk. Baleen whales are long-lived marine predators with relatively low reproductive capacity and tend to make long annual migrations. They may be especially vulnerable to environmental perturbations⁴.

Furthermore, an emphasis on lethal sustainable use may itself help to generate new markets and trade. This already seems

to be the case for caimans⁵ and elephants; the recent CITES decision to resume trade seems to have led to increased poaching. Indeed, several Caribbean states announced in Antigua their wish to start whaling and, on 26 February, three humpback whales were harpooned by aboriginal whalers from Bequia, Grenadines. (The calf was struck first and used alive to lure its mother; a male escort was struck and lost.)

In the light of these recent events, we should like to repeat our call for the establishment of a global whale sanctuary — to protect whales from direct takes in all maritime waters — and seek the support of the scientific community in this endeavour.

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1. Gambell, R. in *Whales, Seals, Fish and Man* (eds. Blix, A. S. et al.) 699–708 (Elsevier Science, 1995).
2. Knauss, J. A. *Ocean Dev. Int. Law* **28**, 79–87 (1997).
3. IWC Chairman's Consultation with Commissioners, Antigua, 3–5 February 1998 (IWC press release).
4. Simmonds, M. P. & Hutchinson, J. D. *The Conservation of Whales and Dolphins — Science & Practice* (Wiley, Chichester, 1996).
5. Brazaitis, P., Watanabe, M. E. & Amato, G. *Sci. Am.* **278**, 70–76 (1998).

No independence for French researchers

Sir — Claude Allègre's intention of "increasing the independence of young researchers" in France is indeed laudable in a country plagued by archaic hierarchical research systems (see *Nature* **392**, 214; 1998). In the current French situation, however, it may well turn out to be an uphill struggle. Allègre's wish goes against a trend towards increasing concentration (and decreasing numbers) of research units that has clearly been favoured by the central administration of the Centre National de la Recherche Scientifique (CNRS) during the past few years.

This has resulted in the elimination of many small but efficient research units that offered good opportunities for young (and not so young) researchers to develop their own research projects without the hindrances inherent in larger bureaucratic entities. This, for example, was my own experience when my research group was squeezed out during the reorganization of Earth sciences research at University Paris 6.

Conversely, this policy has led to the creation of large, heterogeneous conglomerates, which often have little scientific justification but are easier to deal with from a bureaucratic point of view. Under such a system, all researchers, not only young ones, have little or no independence or opportunity to develop really original research projects. The directors of such overblown research 'units' (who may stay in post for as long as 12 years) have absolute control over the funds they receive from CNRS, and therefore wield absolute power. Within such a highly hierarchical system, the so-called "laboratory councils", which are supposed to exercise some control over the scientific policies of research units, are in fact powerless, as their members depend on the goodwill of the director for basic funding.

The problem is further compounded by the fact that most CNRS research units are based in universities, and are often headed by university professors with little time for research. Many of these 'mandarins', as they are usually called in France, have never really accepted the existence of CNRS (an institution founded in 1939!) as an independent research organization, and resent the fact that academic research is no longer exclusively in the hands of university employees.

The result is that CNRS research units are now full not only of young researchers who are debarred from any real responsibility and independence, but also of older 'directors of research' (a CNRS rank which is the equivalent of university

professor) who are not in a position to 'direct' anything or, more accurately, to develop original large-scale research projects. If it is to be turned into a reality, Allègre's wish will have to involve a profound change in CNRS policies.

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Towards European academic union

Sir — Recent articles stressed the problems facing universities in Europe, America and Asia, and emphasized their significance as "knowledge producers of modern economies" (*Nature* **391**, 5–9; 1998). But not all countries are in a position to follow the same model, and each country's special circumstances must be taken into account, as must the lack of academic coherence in some universities.

The latter has severely handicapped the prospects for academic and scientific union in Europe, and has also shaped the history of some universities that are both politically and physically far from Brussels. The Spanish universities of La Laguna and Las Palmas de Gran Canaria in the Canary Islands illustrate the problems faced by academic institutions in peripheral regions of Europe.

With Hawaii and the Galapagos, the Canary Islands are among the most important active, volcanic, natural laboratories in the world. One of the first international references to the archipelago was published in *Nature* more than 100 years ago (Calderon, S. *Nature* **13**, 403–404; 1876) and they are still of great scientific interest. But, despite the socioeconomic importance of the geological resources of the archipelago, geology receives little attention in the islands' academic institutions. There is no specific higher education degree in geology or Earth sciences, and the only department of geology forms part of the faculty of biology.

Given the European Parliament's new capacity to influence scientific and academic policy (see *Nature* **391**, 1; 1998), it is important that these academic imbalances are monitored and corrected. The European Science Foundation has been given the task of examining and advising on research and science policy issues of strategic importance in planning and implementing pan-European initiatives. The foundation could act as the official institution dealing with the relationship between university guidelines, science and

their socioeconomic repercussions. (For example, the natural heritage — tourism, natural parks — of the Canary Islands is crucial for the economy of the archipelago; both appropriate knowledge and scientific supervision are therefore needed.)

Much is said about the new economic, monetary and political union of Europe; perhaps it is time to begin defining the basis of academic and scientific union as well.

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US teaching methods

Sir — The continuously improving performance of the United States in science and technology compared to any other country is contrary to the performance of 17-year-old US children (*Nature* **392**, 5; 1998). This raises the question whether there is a need to change and improve standards in US schools.

The basic approach to teaching is different from that in most other countries. During early education in the United States, emphasis is placed on understanding basic concepts, whereas in other countries the emphasis is on memorizing things. These differences may play an important role in creativity and this may be why, when US children become adult, they outperform the rest of the world.

The recent results of the Third International Mathematics and Science Study are worrying. But, before making any changes in US teaching methods, the long-term advantages of existing teaching methods should be studied. Other factors such as the contribution of foreign-born scientists and money spent on science and technology need also to be considered.

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Correction

The letter headed "Funding and research performance" (*Nature* **392**, 119; 1998) had five signatories: H. F. Moed, M. Luwel, J. A. Houben, H. Van Den Berghe and E. Spruyt. H. Van Den Berghe's name was printed in the wrong format.

The centenarian Golgi apparatus

Paolo Mazzarello and Marina Bentivoglio

One hundred years ago, Camillo Golgi described the cellular apparatus that has since become synonymous with his name. Although its existence was questioned for 50 years, this organelle is now established as the cell's centre for the processing and secretion of proteins.

On 19 April 1898 at the Medico-Surgical Society of Pavia, Camillo Golgi (Fig. 1) presented his finding of a hitherto unknown organelle in Purkinje cerebellar cells (Fig. 2). On the basis of its 'net-like' appearance and intracellular location, he termed¹ this organelle the "internal reticular apparatus". He could hardly have foreseen that, over the next century, his name would become the most frequently mentioned in literature on cellular and molecular biology, and the eponym of the structure he had discovered — the Golgi.

A professor of histology and general pathology at the University of Pavia, Golgi had already acquired an international reputation based on a surprising number of original contributions to biology and medicine².

In 1873, while searching for a recipe that could effectively stain the nervous tissue, he discovered the so-called 'black reaction', known nowadays as Golgi impregnation or Golgi staining. This reaction was based on the use of silver nitrate and potassium bichromate, and it afforded, for the first time, a full view of single nerve cells and their processes.

Aided by his black-reaction method, Golgi analysed several regions of the nervous system in detail, and provided beautiful illustrations of them. Golgi contested the neuron theory (according to which the nervous system is composed of individual cells, like any other tissue), and he believed that the nerve processes stained by his reaction formed a continuous network along which

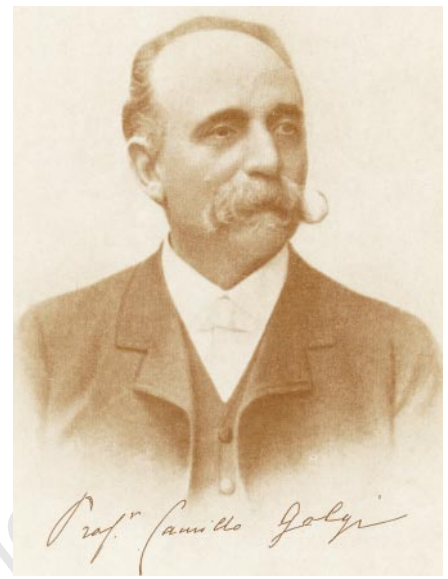


Figure 1 Camillo Golgi (1843–1926) in 1898, the year in which he reported the discovery of the organelle that now bears his name.

the nervous impulse propagated. Ironically, it was by using the Golgi stain that another great neuroanatomist, Santiago Ramón y Cajal (1852–1934), became the paladin of the neuron theory. In 1906, Golgi and Cajal shared the Nobel prize for physiology or medicine, for their investigations into the structure of the nervous system.

Golgi obtained other important results. In 1878, for example, he described the tendinous sensory corpuscles that bear his name (the Golgi tendon organs). Between 1886 and 1892 he concentrated on studying malaria. Not only did he work out the intra-erythrocytic cycle of the malaria agent *Plasmodium*, but he also discovered the temporal relation between the recurrent chills and fever of the infection, and multiplication of the parasite in human blood. However, he never lost his interest in the nervous system.

Using a variant of the black reaction (the rapid method), he had already observed the "internal reticular apparatus" in 1897, in neurons of spinal ganglia (Fig. 2). But he decided to report the existence of this structure only after his findings were replicated and confirmed by one of his students, Emilio Veratti (1872–1967). Working in Golgi's laboratory, Veratti went on to describe, for the first time, the sarcoplasmic reticulum of skeletal muscle fibres in 1902.

In April 1898, Golgi thus felt confident to report¹ that he had observed "a fine and elegant reticulum hidden within the cell body". This was mainly characterized "by the ribbon-like shape of its threads, by their manner of dividing, forming anastomoses, and coursing of these threads... by the presence in tenuous small plaques or small roundish disks transparent at their centre, which serve as nodal points of the reticulum". Soon after the discovery, Golgi's students



Figure 2 Illustrations of Camillo Golgi's "internal reticular apparatus". a, Golgi's first drawing of the apparatus in the body of a Purkinje cell of the cerebellum in 1898. b, The apparatus in spinal ganglion neurons. c, Photomicrograph of the apparatus impregnated by the Golgi reaction (which appears as a black-stained network in the cytoplasm) in spinal ganglia neurons. From an original preparation made at Golgi's laboratory in Pavia. (Part c supplied by V. Vannini, Institute of General Pathology, University of Pavia.)

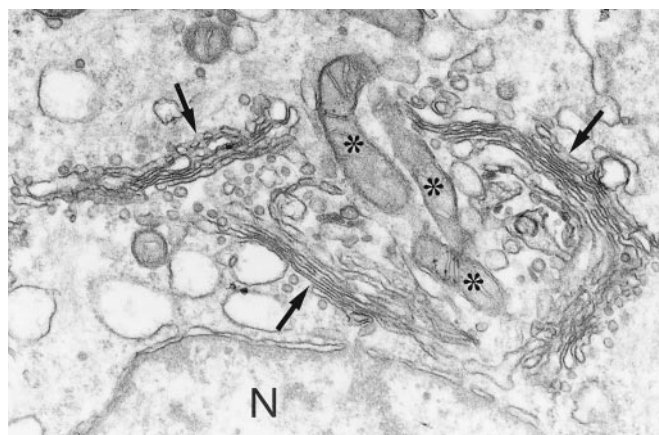


Figure 3 Electron micrograph of stacks of cisternae of the Golgi apparatus (arrows). These are located, as commonly observed, near the cell nucleus (N). The asterisks mark mitochondria.

Antonio Pensa (1874–1970), Adelchi Negri (1876–1912) and Edoardo Gemelli (1878–1959) showed that this structure exists in non-nervous cells as well. (Incidentally, Negri later identified the intraneuronal inclusions that bear his name — Negri bodies — in rabies-infected brains.) Golgi, somewhat timidly, put forward the hypothesis that the apparatus could be involved in secretory functions and, more broadly, in cell nutrition.

The report of this new cell constituent gave a great impetus to cytological studies. In 1913, the internal reticular apparatus was officially christened the Golgi apparatus³. However, the existence of the organelle was debated for decades. The revelatory power of metallic impregnation, for nervous and non-nervous tissues alike, is wonderful when it works. But the capricious and erratic outcomes when this method was applied raised questions about the reality of an intracellular network such as the Golgi apparatus. The debate was inflamed by heated exchanges but, paradoxically, greatly contributed to the scientific pathway through a unifying theory of the cell and its functions.

The controversy was solved only by the introduction of electron microscopy. In 1954, Dalton and Felix⁴, and Sjöstrand and Hanzon⁵ showed that metals are selectively deposited on the membrane and associated vacuoles that make up the Golgi apparatus. Only then did the Golgi apparatus (which was also defined as the Golgi complex) reach the status of a widely accepted cell organelle. Thus, by some sort of historical compensation, the electron microscope — the very instrument that had provided incontrovertible evidence for synapses between neurons, against Golgi's stubborn concept of a continuous interneuronal reticulum — also supplied the final proof that his "internal reticular apparatus" was real.

Over the past few decades, advances such as cell fractionation, histochemistry, autoradiography, immuno-gold labelling in electron microscopy, *in vitro* assays and recombinant DNA technology have unravelled the functions of the cisternae and vesicles that form the Golgi apparatus (Fig. 3). These

include intracellular transport of proteins to the secretory cell surface, intracellular protein sorting, budding and targeting of protein transport vesicles, and viral protein targeting^{6,7}. Research on this key organelle is still blooming.

And what about Camillo Golgi? He would probably have been very surprised and rewarded by the importance of his discovery. But he would have been much less

gratified by the loss of his identity — his name is now just a label for an organelle, or a suffix for its constituents, compartments and functions. Titles that we find nowadays in the scientific literature, such as 'Green light for Golgi traffic'⁸ or 'Greasing the Golgi budding machine'⁹ would probably have raised the professor's eyebrows. □

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1. Golgi, C. *Boll. Soc. Med. Chir. Pavia* **13**, 3–16 (1898); partial transl. Lipsky, N. G. *J. Microsc.* **155**, 3–7 (1989).
2. Mazzarello, P. *La Struttura Nascosta. La Vita di Camillo Golgi* (Cisalpine-Monduzzi, Bologna, 1996; transl. Glickstein, M., Buchtel, H. & Badiani, A., Oxford Univ. Press, in the press).
3. Nussbaum, J. *Arch. Zellforsch.* **10**, 359–367 (1913).
4. Dalton, A. J. & Felix, M. D. *Am. J. Anat.* **94**, 171–207 (1954).
5. Sjöstrand, F. S. & Hanzon, V. *Exp. Cell. Res.* **7**, 415–429 (1954).
6. Rothman, J. E. & Orci, L. *Nature* **355**, 409–415 (1992).
7. Farquhar, M. G. & Palade, G. E. *Trends Cell Biol.* **8**, 2–10 (1998).
8. Pelham, H. R. B. *Nature* **389**, 17–19 (1997).
9. Martin, T. F. J. *Nature* **387**, 21–22 (1997).

Human genetics

Putting the Parkin into Parkinson's

Robert L. Nussbaum

Parkinson's disease is the second most common form of neurodegenerative disease after Alzheimer's¹, affecting 250,000–500,000 people in the United States alone. The disease is characterized by a movement disorder — parkinsonism — which is a triad of rigidity, resting tremor and bradykinesia (slowness in initiating and carrying out movement), often associated with difficulties in maintaining posture. These symptoms result from the dysfunction and loss of neurons that produce the neurotransmitter dopamine, in a part of the brain called the substantia nigra.

On page 605 of this issue, Kitada *et al.*² describe the positional cloning of a previously unknown gene, which they have termed *parkin*. This gene is responsible for a rare autosomal recessive form of parkinsonism, AR-JP, which was discovered^{3,4} and described⁴ by Japanese neurologists. The movement disorder in AR-JP develops in adolescence or young adulthood and responds to levodopa therapy, but it usually progresses and incapacitates the patients after 20–30 years.

At autopsy, brains from patients with AR-JP or Parkinson's disease show loss of neurons in the substantia nigra and locus coeruleus. However, in classical Parkinson's, but not AR-JP, an eosinophilic inclusion body — the Lewy body — is found in the cytoplasm of neurons in the substantia nigra. Lewy bodies react with anti-ubiquitin

antibodies in immunocytochemical studies. They also react with antibodies against α -synuclein, which is a small presynaptic protein of unknown function that is also found in aggregates in ballooned and swollen nerve fibres called Lewy neurites.

The causes of Parkinson's disease are largely unknown, although there are a few rare families with autopsy-proven Lewy bodies in which Parkinson's is inherited in an autosomal dominant manner⁵. The Parkinson's locus has been mapped in some of these families to chromosome 4q, where it encodes α -synuclein⁶. To date, two different mutations in α -synuclein have been found in families with dominant Parkinson's disease^{6,7}. In other families, the locus has been mapped to chromosome 2p13, but the gene itself has not been identified⁸ and, in others still, the gene and its map location remain unknown⁸. In most cases of Parkinson's disease, however, a genetic contribution (if any) remains obscure.

What does AR-JP due to mutations in *parkin* have to do with Parkinson's disease? Parkinsonism can be a feature of many diseases, some hereditary, some acquired, as well as Parkinson's disease itself. Moreover, at first glance, the differences between AR-JP and Parkinson's, rather than the similarities, stand out. AR-JP is an autosomal recessive disorder, whereas the genetic basis for Parkinson's is complex (except for the few rare families in which it is dominantly inher-

ited). AR-JP has no Lewy bodies; Parkinson's disease does. So far, the mutations in AR-JP are deletions which, in accordance with the recessive inheritance pattern, are likely to be loss-of-function mutations (although this remains to be proved). By contrast, in autosomal dominant Parkinson's, only missense mutations have been found in α -synuclein. Their effect on gene function could, therefore, be a partial loss of function, a dominant-negative effect on the product of the normal allele, a toxic gain of function, or some combination of all three.

But there may be more of a connection than is initially apparent. As Kitada *et al.*² point out, Parkin contains structural motifs that provide some hints to its function. One motif is at the amino terminus, where the first 72 amino acids match many different ubiquitin proteins (~33% identity, ~66% similarity). Ubiquitin is a 76-amino-acid peptide which, when covalently linked to a cellular protein and then to itself to form branched polyubiquitin chains, tags the protein for degradation in the 26S proteasome. Lewy bodies are heavily ubiquitinated, and they are immunoreactive with antibodies against neural-specific ubiquitin carboxy-terminal hydrolase, and antibodies against some subunits of the 26S proteasome⁹.

There is currently much speculation as to whether a defect in proteasome function could play a role in Parkinson's and, perhaps, in other neurodegenerative diseases. One hypothesis is that aggregates of abnormal α -synuclein — resulting from an α -synuclein germline mutation, mutations in other genes, or an acquired abnormality — could produce Lewy neurites and trap ubiquitin and proteasome components in Lewy bodies. This would have detrimental consequences to the turnover of neuronal proteins. Parkin, however, lacks two critical residues involved in ubiquitination — a lysine at position 63, and the carboxy-terminal glycine at position 76. Thus, at this point, we do not know if the ubiquitin homology in Parkin indicates that it is even involved in protein degradation, or whether the ubiquitin homology motif is involved in some other way in the function of Parkin. The lack of obvious cellular inclusions in AR-JP speaks against a defect in ubiquitination or proteasome function due to Parkin deficiency.

A second (but not exclusive) hypothesis for the pathogenesis of Parkinson's disease is that the aggregates might themselves be the sites at which other proteins that are important for neuronal function, such as inhibitors of apoptosis, become sequestered and, therefore, unavailable to the cell. It remains to be seen if Parkin is a component of Lewy bodies, and whether its sequestration could lead to a secondary Parkin deficiency and the loss of neurons from the substantia nigra in Parkinson's. Although AR-JP is rare, Kitada *et al.*²

may have identified a previously unrecognized component of what will certainly be a complex pathogenetic pathway leading to Parkinson's disease. □

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1. Tanner, C. M. & Goldman, S. M. *Neurol. Clin.* **14**, 317–335 (1996).
2. Kitada, T. *et al.* *Nature* **392**, 605–608 (1998).
3. Takahashi, H. *et al.* *Neurology* **44**, 437–441 (1994).
4. Ishikawa, A. & Tsuji, S. *Neurology* **47**, 160–166 (1996).
5. Lazzarini, A. M. *et al.* *Neurology* **44**, 499–506 (1994).
6. Polymeropoulos, M. H. *et al.* *Science* **276**, 2045–2047 (1997).
7. Kruger, R. *et al.* *Nature Genet.* **18**, 106–108 (1998).
8. Gasser, T. *et al.* *Nature Genet.* **18**, 262–265 (1998).
9. Mayer, R. J. *et al.* in *Intracellular Protein Catabolism* (eds Suzuki, K. & Bond, J.) 261–269 (Plenum, New York, 1996).

Archaeology

A panorama of ancient Rome

Nicholas Purcell

A remarkable Roman wall-painting was found in the centre of Rome on 4 March by the archaeologist Elisabetta Carnabuci. It is an extremely ambitious cityscape. The building in which it stood is little understood, the wall on which it was painted has not been completely excavated, the painting has not yet been cleaned, and detailed photographs are not yet available. Yet it is not too early to say that this one find vastly increases our store of information about Roman visual ideas of urban space and of public and private architecture.

This is an image of a megalopolis (Fig. 1). The line of the walls alone shows that. Exaggerated in height, and equipped with truly monumental towers, the walls look surprisingly mediaeval in their reminder of the need to defend. But whereas mediaeval city representations often show tightly packed enclosures, here the walls are depicted as impressive in their length, and the urban texture within is loose. The fragment preserved has generous open space as well as densely packed clusters of private buildings which are dominated by enormous public monuments. Two

of these are completely preserved: a marble theatre and a quadrangular precinct, backed by a colonnade, and dominated by a towering central hall. Parts of other such structures are also visible, including one with what appears to be colossal statuary on its roofline, directly beside the theatre.

How specific is this view? Is it a real city? Is it Rome? There are precedents for the angle of view and for the general style. A unique image from Pompeii shows the town itself, and even more surprisingly, a historical event — a famous riot between the Pompeians and their neighbours. The other paintings in this style are images of Roman pleasure-villas on the resort-coasts of southern Italy, and they are usually plausible fantasies. Most of them come from houses near the coastline where real luxury villas stood. The Romans also liked to paint realistic garden scenes beside real gardens. It is interesting to find a painted super-city in a lavish private house in the greatest metropolis of the ancient world. Nearby in Rome we know of other decorative schemes which played with views of the city and out of the city, juxtaposing town and



Figure 1 The newly discovered Roman wall painting. A line of fortifications forms the backdrop to the view, with a monumental bridge beyond (top left). To the left we see the white marble curve of a theatre and its auditorium; to the right a public monument. A detailed disorder of private buildings runs across the front of the scene.

country in real vistas and artistic representation. It seems likely that this is a further example of that taste.

Reports have associated the new painting with the Emperor Nero (AD 54–68), whose lavish themed palace, the Golden House, was nearby. It has even been suggested that this is Rome before the destructive Great Fire of AD 64, which was blamed on Nero, and which made possible the building of his dream home by obliterating the earlier urban landscape. In fact, the painting is probably much later.

There are other reasons, though, to link it, at least impressionistically, with the urban ideal of Rome as the capital, and wonder, of the world. In the background is a large river crossed by a monumental bridge, not a common feature of ancient cities. And there are

not many ancient cities that could claim such extremes of metropolitan grandeur — Alexandria, Antioch, Carthage; the list is not long. But the pictured city is hard to relate to the topography of ancient Rome, which did not acquire walls like this until the end of the third century AD. Neither does anything in the picture as it survives especially suggest the features we know of other great Roman cities. It is better to take it as a different kind of image — but one that is just as fascinating, for the insight it offers into how people of two millennia ago perceived urban environments that were as complex, socially and culturally, as any seen again in Europe before the nineteenth century. □

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Developmental biology

Worlds in common through NF- κ B

Cheryll Tickle

A transcription factor that regulates cell activity in the immune system and a human syndrome that affects face and limb development seem worlds apart. On pages 611 and 615 of this issue, however, Kanegae *et al.*¹ and Bushdid *et al.*² report an unsuspected role of NF- κ B that brings these worlds together in the context of chick limb development.

NF- κ B (or, to give it its full name, nuclear factor regulating expression of kappa light-chain immunoglobulin) is a well-known transcriptional regulator that was first identified in B cells³. Its activity is mediated by a family of molecules, including the product of the gene, *c-rel*. The two new papers^{1,2} report that *c-rel* is expressed in cells at the tip of chick limb buds. When NF- κ B activity is blocked, limb abnormalities, mostly truncations, are observed. This provides direct evidence that this intracellular signalling mechanism is necessary for vertebrate embryonic development. Members of the NF- κ B family were already known to be homologous to the product of the *Drosophila* gene *Dorsal*⁴, and this led to the identification of targets of NF- κ B signalling in the chick limb. Last year, mutations in one of these targets, the *Twist* gene, were shown to be responsible for the human Saethre-Chotzen syndrome in which limb anomalies are found⁵.

The region of undifferentiated mesoderm cells in the chick limb bud that expresses *c-rel*, and thus possesses NF- κ B activity, is where patterning signals operate. This region is called the progress zone and remains at the tip of the bud as it grows out. As cells leave this zone, they form the series of structures along the long axis of the limb in succession — for example, first 'upper arm', then 'lower arm' and finally 'hand'. The

current working hypothesis is that limb cells 'know' which structure to form by the length of time they spend in the progress zone⁶.

Almost exactly 50 years ago, John Saunders⁷ discovered that the thickened rim of the epithelial covering of the limb bud, the apical ectodermal ridge, is responsible for maintaining the progress zone. When the ridge is cut away, limb truncations result. The extent of truncation depends on the stage of limb development at which the ridge is removed, and ranges from almost complete absence of limb (ridge removed early) to just absence of the hand (ridge removed late). Truncations are the most consistent type of defect reported by Kanegae *et al.*¹ and Bushdid *et al.*² when NF- κ B signalling is inhibited, although sometimes the limbs are also 'twisted'. Interactions between ridge and progress zone are reciprocal, and cells in the progress zone in turn maintain the ridge. So blocking NF- κ B function may interfere either with the response of progress zone to ridge signalling or with progress zone signalling to the ridge.

The intimate knowledge of how NF- κ B operates provided a neat way of demonstrating *c-rel* function in the chick limb. In unstimulated cells, NF- κ B is sequestered in the cytoplasm by the inhibitory protein I- κ B (Fig. 1a, overleaf). When the cell is stimulated, NF- κ B is released and moves from cytoplasm to nucleus, where it binds to regulatory elements of target genes. Signalling can therefore be blocked by mutant forms of I- κ B which do not release NF- κ B. Such forms of I- κ B, for example a form that cannot be phosphorylated and degraded, were expressed by Kanegae *et al.* in the developing chick limb using viruses, and this led to the limb truncations. Mice in which the gene encoding NF- κ B has been knocked out



100 YEARS AGO

I send you a photograph of probably the most extraordinary heron's nest ever discovered in this or any other country. During a gale it was blown from the top of an elm tree in the heronry on Stoke Hall estate in Notts, the seat of Sir Henry Bromley, Bart. It is of unusual size, and almost exclusively composed of wire of varying lengths and thickness; the centre, or "cup," alone being composed of fine twigs, grasses and feathers. ... The other curious feature of the Stoke Hall phenomenon is that there is not, and never has been, any lack of ordinary building material, and that all the wire used must have been carried a great distance.

An interesting observation upon the development of a taste for honey by starlings is recorded by Mr. W. W. Smith in the *Entomologist* (April). In a previous note referring to some enemies of humble-bees in New Zealand, Mr. Smith stated that he had observed the newly-introduced starlings killing and conveying humble-bees to their nests to feed their young. ... this bird now frequents the flax-flats and sucks the honey from the richly mellifluous flowers. It appears probable that the eating of the humble-bee's honey-sac by the starlings developed, or is now developing, the taste for honey in these birds.
From *Nature* 7 April 1898.

50 YEARS AGO

In 1941 we published a theory which provided, among other things, an explanation of seasonal and latitude variations in the thickness of atmospheric ozone. From this theory we were able to predict ozone thicknesses in latitudes for which, as yet, there are no direct observations — for example, in polar regions. Furthermore we have shown that near the poles the ozone thickness should be practically zero soon after the winter solstice. Now routine measurements of ozone thickness, carried out by E. Tönsberg and K. L. Olsen in Tromsø, have shown values so low as 0.05 cm. in December. It may be assumed that these occurrences are due to the movement of air masses from higher latitudes, so that the results afford good confirmation of our theoretical predictions.
From *Nature* 10 April 1948.

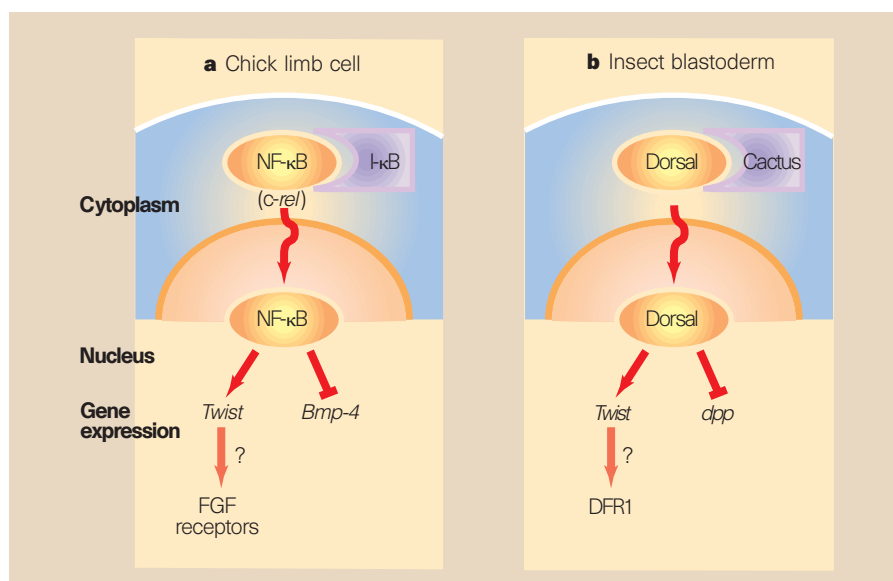


Figure 1 Outline of a developmental pathway based on NF- κ B signalling. **a**, Signalling through NF- κ B in chick limb cells occurs by its release from I- κ B and translocation to the nucleus where it regulates target genes (activation of *Twist* and repression of *Bmp-4*). Gene(s) encoding receptors for fibroblast growth factor (FGF) could lie downstream of *Twist*. These targets in the chick limb bud cells were identified by Kanegae *et al.*¹ and Bushdid *et al.*² by reference to known targets of NF- κ B in *Drosophila*. **b**, In *Drosophila* blastoderm, signalling through Dorsal, a homologue of NF- κ B, occurs by its release from Cactus and translocation to the nucleus, where it activates *Twist* and represses expression of *decapentaplegic* (*dpp*, of which *Bmp-2* and *Bmp-4* are vertebrate homologues). A *Drosophila* homologue of vertebrate FGF receptors (DFR1) appears to lie downstream of *Twist*, at least in the imaginal discs. A human connection comes from the finding³ that mutations in the *Twist* gene cause Saethre–Chotzen syndrome, which is characterized by limb anomalies. For simplicity, NF- κ B activity is represented here as a single entity, although it is known to act as a hetero- or homo-dimeric complex.

develop normally, however, presumably because other NF- κ B family members compensate for its absence⁸.

Further dissection of the NF- κ B signalling pathway in the chick limb was possible

because of the extraordinary parallels between development in *Drosophila* and in vertebrates. NF- κ B is homologous to the product of the *Drosophila* gene *Dorsal* — *Dorsal* signalling occurs by the same mecha-

nism as NF- κ B signalling, with Cactus being the homologue of I- κ B (Fig. 1b)⁹. *Dorsal* is known to activate expression of a gene called *Twist* (encoding a regulatory helix–loop–helix protein), in the region of the insect body where mesoderm will form.

This led Kanegae *et al.*¹ and Bushdid *et al.*² to examine *Twist* expression in the chick limb, and they show that, when NF- κ B signalling is blocked, *Twist* expression is reduced. Interestingly, early limb buds of *Twist*-null mouse embryos, which die about midway through gestation, have been reported to be stunted¹⁰. This then fits very nicely with the limb truncations obtained when NF- κ B signalling is blocked. Another insight from *Drosophila* links *Twist* expression to apical ridge signalling. In *Drosophila*, a gene closely related to the genes encoding receptors for vertebrate fibroblast growth factor appears to be downstream of *Twist*⁵. Apical ridge signalling in the chick limb is known to be mediated by fibroblast growth factors. So *Twist* expression in the limb could be directly connected with the ability of cells in the progress zone to respond to ridge signals.

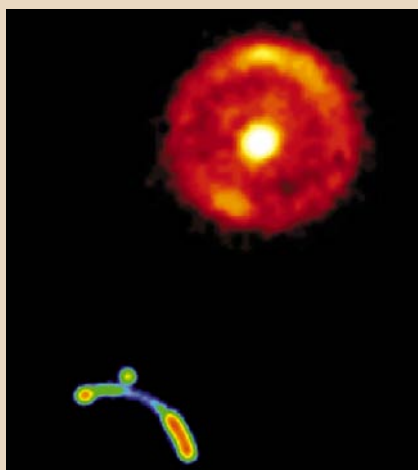
In *Drosophila*, *Dorsal* not only promotes *Twist* expression but also represses expression of *decapentaplegic* (*dpp*), which encodes a signalling molecule (Fig. 1b). Again, a parallel emerges in the chick limb. Bushdid *et al.*² show that blocking NF- κ B signalling leads to increased expression of a vertebrate homologue of *dpp*, a gene encoding a bone morphogenetic protein, BMP-4. These proteins were originally named because of their ability to induce bone. They now appear to be multifunctional molecules, being implicated not only in skeletal

Extragalactic astronomy

A ring in truth

The orange bullseye is an image of two galaxies. One, at the centre, has stretched the image of the other into a ring — the first complete ‘Einstein ring’ ever seen. This is just one of many gravitational lenses collected in recent years, by a programme that aims to answer fundamental questions about the Universe’s geometry and composition.

Einstein’s theory of General Relativity predicts that light rays are curved by gravitational fields. The first confirmation of this effect came in 1919, when the Hyades star cluster was observed slightly out of place during a solar eclipse — a weak example of gravitational lensing. More interesting lenses occur where one distant galaxy or quasar appears to us in several distorted images, around the position of an intervening galaxy or galaxy cluster. More than 20 are known, including some partial Einstein rings, where the



lensed and the lensing objects are almost perfectly aligned.

Prompted by observations from the MERLIN array of radio telescopes, the Hubble Space Telescope has imaged this

complete Einstein ring (L. J. King *et al.* *Mon. Not. R. Astron. Soc.* 295, L41–L44; 1998). The radio image, shown below Hubble’s infrared image, isn’t a complete ring because the radio-emitting part of the lensed galaxy is a little off-centre.

So how do lenses reveal the geometry of the Universe? It is a matter of statistics. The redshift distribution of lensed objects tells us what proportion of our lines of sight to distant regions are blocked by intervening galaxies. That is a function of the geometry of space-time, which in turn depends on the amount of matter present, and on the energy density of empty space — the ‘cosmological constant’, which Einstein introduced and then abandoned, but which is beginning to come into vogue again. By the end of this year, the team working with MERLIN hope to put the strictest limits yet on the cosmological constant.

Stephen Battersby

differentiation in the chick limb but also in positional signalling and programmed cell death¹¹.

The connection between NF- κ B and *Twist* provides the satisfying link to Saethre-Chotzen syndrome. This human condition is characterized by craniofacial and limb anomalies, and the same developmental pathway probably operates in both regions of vertebrate embryos. At present, it is difficult to see just how alterations in *Twist* could lead to the specific anomalies in the limb (short digits and soft tissue webbing between digits). For although genes bring different worlds together, the challenge remains to understand how gene expression is translated into anatomy. □

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1. Kanegae, Y., Tavares, A. T., Izpisua Belmonte, J. C. & Verma, I. M. *Nature* **392**, 611–614 (1998).
2. Bushdid, P. B. et al. *Nature* **392**, 615–618 (1998).
3. Liou, H.-C. & Baltimore, D. *Curr. Opin. Cell Biol.* **5**, 477–487 (1993).
4. Steward, R. *Science* **238**, 692–694 (1987).
5. Dixon, M. J. *Nature Genet.* **15**, 3–4 (1997).
6. Summerbell, D., Lewis, J. & Wolpert, L. *Nature* **224**, 492–496 (1973).
7. Saunders, J. W. J. *Exp. Zool.* **108**, 363–404 (1948).
8. Schmidt-Ullrich, R. *Development* **122**, 2117–2128 (1996).
9. Wolpert, L. *Principles of Development* 135 (Current Biology, Oxford, 1998).
10. Chen, Z.-F. & Behringer, R. R. *Genes Dev.* **9**, 686–699 (1995).
11. Johnson, R. L. & Tabin, C. J. *Cell* **90**, 979–990 (1997).

Computing

Parallel thinking

C. S. Calude and J. L. Casti

The computer seems to be the only commodity ever to become exponentially better as it gets cheaper. Its information handling capacity has grown at a rate ten million times faster than that of our nervous systems during the four billion years since life began on Earth. Yet the theory and technology of computing has rested for more than 50 years on the Turing-machine model of computation, which leads to many intractable or undecidable problems. Are there alternatives? This was the question addressed at a conference in January*, where three new models of computation were discussed: the DNA model, the quantum model and the reversible model.

DNA has a potentially gigantic memory capacity (in reasonable concentrations, a litre of DNA solution can store up to 10^{22} bits of information), and biochemical operations are massively parallel. So DNA has a built-in computational power. The familiar double helix of DNA arises by the bonding of two separate polymer chains, composed of the four DNA bases A, G, C and T. These obey the Watson–Crick complementarity rule: A bonds with T and C bonds with G. This restriction means that one DNA chain can pair with another chain only when their sequences of bases are complementary. Thus, fundamental information is available for free: knowing one member of a bond means automatically knowing the other.

The startling thing is that complementarity yields universality, in the Turing sense (A. Salomaa, Turku Univ.). Consider the set of all possible words (sequences) that can be obtained from two given words by shuffling them without changing the order of letters. For instance, shuffling AG and TC we get AGTC, ATCG, TCAG and TAGC. Then col-

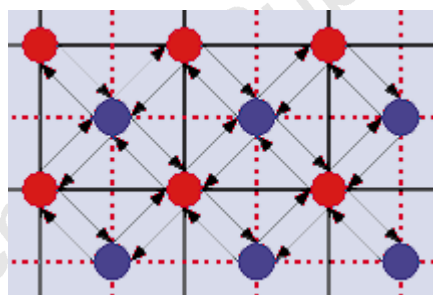


Figure 1 The layout of a single cell of 'Flattop', an adiabatic (and therefore reversible) processor. It is based on the billiard-ball cellular automaton, in which logic values are modelled by the presence or absence of 'billiard balls' moving along paths in a grid. This scheme has now been realized, using a split-level charge-recovery logic circuit.

lect all shufflings of all pairs of complementary words into the so-called twin-shuffle language. There is a simple way to go from a DNA double strand to a word in the twin-shuffle language and back. Universality follows from the fact that any Turing computation can be performed by using an appropriate finite automaton to filter the (fixed) twin-shuffle language. So DNA computers could in theory perform any operation that digital computers can.

It is a long way from theory to implementation, however. Biochemical operations are slow and prone to errors. The extraction of DNA strands containing a particular sequence is far from certain. Physical constraints, such as volume (performing the computation within a practical volume of DNA), time (some operations can take up to 100 minutes, at present) and energy (operations such as denaturing and annealing require heating or cooling) are difficult to control. But a new cloning readout procedure that overcomes some of these problems was

described at the meeting. Using bacteriophage DNA rather than synthesized DNA to carry encoded solutions, operations such as removal, restriction and sorting can be performed by gel electrophoresis, and the result can be obtained in an error-resistant way by picking individual clones and sequencing their DNA (M. Amos, Liverpool Univ.).

Computers, in contrast to Turing machines, are physical devices: whatever they can or cannot do is determined by the laws of physics. Quantum effects, such as interference and entanglement, are especially important, because in the race for miniaturization, computers will inevitably use circuits approaching the level of atoms and photons. There are also certain algorithms that, in principle, can be solved by a quantum computer much more quickly than by a conventional one.

Not all quantum effects may be needed. Nonlinearity (to support quantum logic and ensure universality) and coherence (for the manipulation of coherent quantum superpositions) are necessary and, in principle, sufficient conditions for computation. Conventional devices under investigation for carrying out these operations include ion traps, high-finesse cavities for manipulating light and atoms using quantum electrodynamics, and molecular systems designed to compute using nuclear magnetic resonance. These last store quantum information in the states of quantum systems such as photons, atoms or nuclei, and realize quantum logic by semiclassical potentials such as microwave or laser fields. Unconventional ideas for quantum computation include fermionic quantum computers, bosonic computers (which use a Bose–Einstein condensate of photons, phonons or atoms), and architectures relying on anyons (whose nonlocal topological nature makes them intrinsically error-correcting and virtually immune to noise and interference).

The third strand of the meeting was reversibility. An operation is reversible if it can be undone; it is simply determinism looking backwards in time. Conventional computers are irreversible, and constantly discard information about their states. This limits their efficiency, as it increases the energy required to perform computations and involves the dissipation of heat. But since the laws of physics are reversible at a microscopic level (a given microstate can only be reached by a single path), it follows that irreversible operations and the accompanying production of entropy are in principle not necessary. In practice, reversible computations are likely to dissipate far less energy — but avoiding all entropy production may hurt other measures of performance, such as speed.

The world's first fully reversible universal computer was presented at the meeting (T. Knight, MIT). Working with a parallel architecture (Fig. 1), the machine can perform any computation using arbitrarily little energy per operation (ignoring leakage and power-

*Unconventional Models of Computation, Univ. Auckland, New Zealand, 5–9 January 1998.

supply issues). Even with actual leakage factors taken into consideration, the machine operates with less than one-thousandth of the energy per operation of a conventional circuit implementing the same computational model.

Although all these techniques are becoming more tractable, there is still no sign of any way to break the Turing barrier. Which computational route holds the most promise for the future? In spite of profound differences — in philosophy, methodology, resources — all face a number of similar problems. How should we efficiently encode the data? What architecture should we choose? How is the architecture initially programmed? Can the architecture reconfigure itself? How should we modify the presently known algorithms to fit the new architecture? How should we efficiently read the output?

So it is a matter of some debate whether any of these models will ever leave the lab. But they have helped draw together the disci-

plines of computing, mathematics, physics, engineering and biology, and already produced new insight. For instance, new ideas have arisen about the evolution of genes and DNA sequences (that life may be seen as a series of complex computations) and in the field of quantum communication; and solutions have been obtained to old problems (for example, the negative solution to Maxwell's demon problem (S. Lloyd, MIT): a perfectly efficient engine is impossible not only for mortals, but even in principle). The real issue might not be the final destination, but the journey, and the understanding of natural phenomena that will necessarily occur along the way. □

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Ozone depletion

A greenhouse warming connection

Ross J. Salawitch

On page 589 of this issue¹, Shindell and colleagues tackle the question of how rising levels of greenhouse gases will affect ozone (O₃) depletion over the Arctic in the years to come. Their conclusion is tentative but worrisome — that despite reductions in emissions of O₃-depleting chlorofluorocarbons (CFCs), levels of O₃ will continue to fall, with especially severe declines occurring in the years 2010 to 2019.

The direct radiative effects of the build-up of carbon dioxide (CO₂) and other greenhouse gases have led to a gradual cooling of the stratosphere, with the largest changes in temperature occurring in the upper stratosphere, well above the region of peak O₃ concentrations². Build-up of greenhouse gases has been associated with global warming, which occurs as more of the heat radiated from Earth's surface is trapped in the troposphere; as a consequence, less heat reaches the overlying stratosphere, which cools. Cooling of the upper stratosphere during the past several decades would lead to slightly higher concentrations of O₃ due to the temperature dependence of the rates of several key reactions. But this effect has been masked by depletion of upper stratospheric O₃ driven by the release of industrial CFCs (ref. 3). It has also been suggested that the abundance of O₃ in the lower stratosphere may be reduced on longer timescales due to changes in circulation induced by the so-called 'doubled CO₂' environment⁴. Because the greatest concentrations of O₃ exist in the lower stratosphere, this region has the

strongest influence on the total column abundance of O₃ (the integrated amount of O₃ between the surface and the top of the atmosphere), which in turn affects exposure to ultraviolet radiation.

Shindell *et al.* now describe a previously unappreciated connection between greenhouse gases and O₃ for the contemporary lower stratosphere. They use a general circulation model (GCM) to show that increasing concentrations of greenhouse gases may currently be leading to colder, more stable vortex circulations in winter, accelerating the chemical removal of O₃ at high latitudes. The authors' calculations suggest that, because of the build-up of greenhouse gases, the total column abundance of O₃ in the Arctic vortex will continue to decrease for about 15 years after levels of stratospheric chlorine begin to decline.

Rapid loss of O₃ throughout the winter polar vortices occurs under conditions of high concentrations of chlorine monoxide (ClO), low temperatures and relatively long periods of daylight. The unreactive reservoirs, HCl and ClNO₂, are converted to ClO by reactions on the surfaces of polar stratospheric clouds that form when temperature drops below a critical threshold. Sunlight is required for catalytic removal of O₃, but sunlight also leads to the suppression of increased concentrations of ClO. Maintenance of high concentrations of ClO until the equinox, when day and night are of equal duration and rates of O₃ loss tend to maximize in the Antarctic, requires either temperatures persistently low enough to

Figure 1 Ozone levels and minimum temperature over the Arctic (63 to 90° N), 1979–98. a, Time series of the average total ozone column during March (from ref. 6). Column O₃ data for 1998 are from Earth Probe TOMS. (One Dobson unit (DU) is the thickness, measured in units of hundredths of a millimetre, that the ozone column would occupy at standard temperature and pressure.) b, Minimum temperature on the 50-mbar pressure level in the Arctic vortex during the first two weeks of March, based on data from the National Center for Environmental Prediction; the temperature threshold for formation of polar stratospheric clouds is also shown. c, Scatter diagram of these observations, with the data labelled according to the year of observation. Shindell *et al.*¹ show that rising concentrations of greenhouse gases may be the cause of the colder conditions in recent years, accelerating the chemical loss of O₃ by ClO derived from industrial CFCs. Observations for 1998 are preliminary and will be the subject of a future publication by P. A. Newman and R. D. McPeters. I thank them for making these observations available for inclusion in this figure.

suppress concentrations of gas-phase HNO₃, which photolyses and eventually converts ClO to ClNO₂, or temperatures intermittently low enough to allow repeated exposure of air to heterogeneous processing on

polar stratospheric clouds. More stable vortex circulations (that is, greater isolation) typically lead to colder temperatures and a stronger association between local chemical loss and the column abundance of O₃.

Ozone concentrations in the Arctic polar vortex reached unusually low values during the early springs of 1996 and 1997 (refs 5–7). Compared to the Antarctic vortex, which experiences elevated concentrations of ClO until the equinox and massive loss of O₃ on an annual basis, concentrations of ClO in the Arctic vortex are usually too low by early March for sustained rapid loss of O₃ (ref. 8). The rapid loss of Arctic ozone during late winter and early spring of 1995–96 and 1996–97 has been associated with observations of higher levels of ClO (ref. 9), suppressed levels of HCl (ref. 5), unusually low temperatures during early March^{5–7,10}, and more stable vortex circulations¹⁰. Conditions during these two years have built upon the general (but not monotonic) trend in the Arctic towards lower abundances of total column O₃ in March and lower temperatures in late February and early March during recent years, as illustrated in Fig. 1, even though the burden of stratospheric chlorine has stopped rising because of international legislation that has phased out the use of CFCs (ref. 11).

Shindell *et al.*¹ provide a provocative explanation for the unusually cold, stable Arctic vortices during early March in recent years — a decrease in the poleward propagation of planetary waves, driven by increased concentrations of greenhouse gases. The decrease in planetary-wave activity in their model results from a decline in the latitudinal temperature gradient near the tropopause. A feedback involving decreased absorption of solar radiation due to less O₃ exacerbates the situation, leading to a non-linear response of temperature to climate forcing. An analysis of temperature fields from the National Center for Environmental Prediction has shown that the low Arctic temperatures during March 1997 were probably due to a significant reduction in planetary-wave activity¹⁰. This hypothesis may also offer an explanation for the rapid acceleration in the severity of the Antarctic O₃ hole during the 1980s.

Shindell and colleagues' hypothesis must be viewed as somewhat speculative, given the difficulty GCMs have in properly simulating both temperatures in the polar region and the nonlinear wave-wave interactions that lead to poleward transport of heat and momentum^{4,12}. Reliable predictions of stratospheric wave transport are critically dependent on the proper simulation of the circulation of the upper troposphere^{4,12,13}. The detailed dynamical conditions that lead to low temperatures within the Arctic vortex vary from year to year^{7,10,13,14} and are inherently difficult to simulate using a GCM.

Indeed, the primary conclusion of Shindell *et al.* is model dependent: some but not all other GCMs find similar decreases in planetary-wave activity as the concentrations of greenhouse gases rise¹. Nonetheless, during the past decade there has been an apparent trend towards colder conditions later in the season^{6,7,10,14}, lower abundances of total column O₃ (ref. 6), and more extensive chemical removal of lower stratospheric O₃ (refs 5, 6, 9) that resembles the predictions of Shindell and colleagues.

The hallmark of the Arctic vortex is large year-to-year variability in temperature, the concentration of ClO in February and March, and the degree of chemical loss of O₃. The Arctic winter of 1997–98 has been fairly warm, with considerably less chemical loss of O₃ than occurred in the previous five winters (G. L. Manney, P. A. Newman & M. L. Santee, personal communication). This behaviour is not necessarily inconsistent with the model of Shindell *et al.*¹, which predicts less frequent early warmings of the Arctic vortex but not the complete cessation of such events. However, the winter of 1997–98 certainly complicates ascribing a climatic influence to the changes in temperature and O₃ that have occurred during the previous five winters. Predicting the future course of both

Antarctic and Arctic O₃ is contingent upon gaining a better understanding of the factors that regulate the temperature of the polar vortices, and it is imperative that the hypothesis of Shindell *et al.* be tested by further analyses of atmospheric observations as well as by additional theoretical studies. □

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1. Shindell, D. T., Rind, D. & Loneragan, P. *Nature* **392**, 589–592 (1998).
2. Ramaswamy, V. & Bowen, M. M. *J. Geophys. Res.* **99**, 18909–18921 (1994).
3. Jackman, C. H., Fleming, E. L., Chandra, S., Considine, D. B. & Rosenfield, J. E. *J. Geophys. Res.* **101**, 28753–28767 (1996).
4. Austin, J. & Butchart, N. *J. Geophys. Res.* **99**, 1127–1145 (1994).
5. Müller, R. *et al.* *Nature* **389**, 709–712 (1997).
6. Newman, P. A., Gleason, J. F., McPeters, R. D. & Stolarski, R. S. *Geophys. Res. Lett.* **24**, 2689–2692 (1997).
7. Manney, G. L., Froidevaux, L., Santee, M. L., Zurek, R. W. & Waters, J. W. *Geophys. Res. Lett.* **24**, 2697–2700 (1997).
8. Salawitch, R. J. *et al.* *Science* **261**, 1146–1149 (1993).
9. Santee, M. L., Manney, G. L., Froidevaux, L., Zurek, R. W. & Waters, J. W. *Geophys. Res. Lett.* **24**, 2713–2716 (1997).
10. Coy, L., Nash, E. R. & Newman, P. A. *Geophys. Res. Lett.* **24**, 2693–2696 (1997).
11. Montzka, S. A. *et al.* *Science* **272**, 1318–1322 (1996).
12. Hamilton, K. *Clim. Dynam.* **11**, 223–241 (1995).
13. Austin, J. & Butchart, N. *J. Geophys. Res.* **97**, 10165–10186 (1992).
14. Naujokat, B. & Pawson, S. *Geophys. Res. Lett.* **23**, 3703–3706 (1996).

Evolutionary ecology

Different routes to similar ends

Paul H. Harvey and Linda Partridge

In science, it can be all too easy to provide the public with different take-home messages from the same body of theory and data. Nowhere is that more apparent than from evolution, where different authors have championed different perspectives.

Richard Dawkins, for example, frequently emphasizes evolution by natural selection as a unifying theory for biology: his is a largely deterministic world following from the differential survival and reproductive success of genes within which there is no recombination¹. By contrast, Stephen Gould concentrates on the evidence for biodiversity resulting from contingency — historical accidents determine disparate courses of evolution². In his metaphor, if the tape of life was repeated, we should always expect a different outcome.

These perspectives leave open the middle ground: if the tape of life was repeated, just how varied would the outcome be? In a paper published in *Science* last month³, Jonathan Losos and colleagues describe their studies of *Anolis* lizard species evolving on the Caribbean islands of the Greater Antilles to provide an illuminating test case.

Four islands constitute the Greater Antilles: Cuba, Hispaniola, Jamaica and Puerto Rico. Each of these islands has its

own *Anolis* community. Working over four decades, Ernest Williams and his students demonstrated that each species on each island has a distinctive niche^{4,5} to which it seems to be nicely adapted^{6,7}. There are small, short-legged species that live out on fragile twigs, and large-bodied species that inhabit the crowns of trees; and there are others again on the trunk, on the ground and in bushes, and on transitional habitats. Williams called these habitat specialists 'ecomorphs'⁸. Losos has identified six ecomorphs (examples are shown in Fig. 1), most of which are present on each island; two are missing from Jamaica and one from Puerto Rico. Some islands have more than one version of a particular ecomorph.

But are the ecomorphs figments of Williams's and Losos's imaginations, or do the same ecomorphs from different islands cluster together in 'morphospace'? In the new study, a variety of morphological characters was measured on several specimens from each ecomorph on the different islands, corrected for body size, and mapped in four-dimensional morphospace, which accounted for more than 95% of variance in the data. The data were then subjected to a hierarchical classification by similarity, and each of 46 species was found to group by ecomorph,



Figure 1 Examples of the ecomorphs of *Anolis* lizards studied by Losos *et al.*³. Above, a typical trunk-ground ecomorph, this one from Hispaniola. Top right, the twig ecomorph from Hispaniola, with (beneath it) the convergent twig ecomorphs from Jamaica and Puerto Rico.

independently of its island of origin.

Parsimony arguments based on morphological evolution might lead us to conclude that each ecomorph had a single evolutionary origin, followed by dispersal among the islands. Given six ecomorphs, there would have been five morphological transitions over evolutionary time. Alternatively, similar ecomorphs could have evolved independently on the four islands. Under the conservative assumption that each ecomorph evolved at most once on each island, there would have been between 17 and 19 evolutionary morphological transitions (depending on which was the ancestral phenotype). To distinguish between these two extremes, Losos *et al.* produced a molecular phylogeny based on mitochondrial DNA sequences relating the various species from the different islands. The most parsimonious tree based on molecular evolution, and all trees within four molecular steps of it, required 17 morphological transitions, as did maximum-likelihood trees.

In fact, the molecular phylogenetic analysis identifies just two cases where the same ecomorph has evolved twice on an island, which makes the occurrence a rare event. Losos *et al.* suggest that once an ecomorph has evolved on an island it tends to fill the available niche, preventing other species evolving into it. It seems then that as the tape of life has been replayed on the separate islands, there has been a remarkable amount of convergent evolution.

But there remains a role for contingency: the order of evolution of the different ecomorphs seems to differ among the islands. Four ecomorphs are common to each of the four islands, but no phylogenetic tree relating the four ecomorphs is common to any pair of islands (contrary to preliminary evidence reported earlier)⁶. For example, on Puerto Rico the trunk-crown ecomorph's closest relative is the trunk-ground, whereas on Jamaica it is the crown-giant, and on Cuba it is the twig ecomorph. It is not cur-



rently possible to distinguish between two extreme causes for these differences. The same end state may have been reached from either similar or different starting conditions, with the same ecomorph or different

ecomorphs initially invading each island.

Perhaps evolutionary biologists should not be surprised at such convincing evidence for convergent morphological evolution. After all, the comparative method, which was used so effectively by Darwin and has been a cornerstone of evolutionary investigation ever since, depends for its very existence on repeated instances of convergent evolution⁹. More novel is the finding that the order of evolutionary transitions can differ so markedly — it does not seem to matter what route you take, you'll get there in the end. □

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1. Dawkins, R. *Climbing Mount Improbable* (Viking, London, 1996).
2. Gould, S. J. *Wonderful Life* (Norton, New York, 1989).
3. Losos, J. B., Jackman, T. R., Larson, A., de Queiroz, K. & Rodríguez-Schettino, L. *Science* **279**, 2115–2118 (1998).
4. Rand, A. S. & Williams, E. E. *Breviora* **327**, 1–19 (1969).
5. Williams, E. E. in *Lizard Ecology: Studies of a Model Organism* (eds Huey, R. B., Pianka, E. R. & Schoener, T. W.) 326–370 (Harvard Univ. Press, Cambridge, MA, 1983).
6. Losos, J. B. in *New Uses for New Phylogenies* (eds Harvey, P. H., Leigh Brown, A. J., Maynard Smith, J. & Nee, S.) 308–321 (Oxford Univ. Press, 1996).
7. Losos, J. B. & Irschick, D. J. *Anim. Behav.* **51**, 593–602 (1996).
8. Williams, E. E. *Evol. Biol.* **6**, 47–89 (1972).
9. Harvey, P. H. & Pagel, M. D. *The Comparative Method in Evolutionary Biology* (Oxford Univ. Press, 1991).

Signal transduction

Taking the Rap

Christopher J. Marshall

Treatment of the phaeochromocytoma cell line PC12 with nerve growth factor (NGF) leads to differentiation of the cells into a phenotype that resembles sympathetic neurons. Yet treatment with other growth factors, such as epidermal growth factor (EGF), does not. How is such specificity in growth-factor signalling achieved?

On page 622 of this issue, York *et al.*¹ provide a clue. NGF-mediated differentiation is known to require signalling by Ras (ref. 2), and the authors now show that another small GTPase, Rap1, is also involved. Together with other studies from the same group³, these results indicate that, as well as the well-characterized Ras pathway, there is another pathway involved in NGF-mediated signalling.

There is considerable evidence that differentiation of PC12 cells requires sustained activation of the extracellular-signal-regulated kinase (ERK) pathway, also known as the mitogen-activated protein (MAP) kinase pathway. NGF-stimulated activation of these

kinases is maintained for several hours. By contrast, treatment of wild-type PC12 cells with EGF — which does not cause the cells to differentiate — leads to transient activation of the ERK MAP kinases. If PC12 cells are engineered to overexpress EGF receptors, there is prolonged activation of ERKs and, as a result, the cells differentiate in response to EGF (ref. 4). Conversely, when activation of ERK is blocked by treatment with a synthetic inhibitor⁵ of MEK (the kinase that normally activates ERK; Fig. 1, overleaf), NGF-mediated differentiation is blocked. An attractive model to account for the different consequences of transient versus sustained activation of ERKs is that sustained activation leads to persistent nuclear accumulation of ERKs, resulting in phosphorylation of transcription factors and changes in gene expression⁶.

The ERK/MAP kinases are switched on by receptor tyrosine kinases, through activation of the small GTPase Ras. Activated Ras binds to Raf-1, recruiting it to the plasma membrane where it is activated; then Raf-1

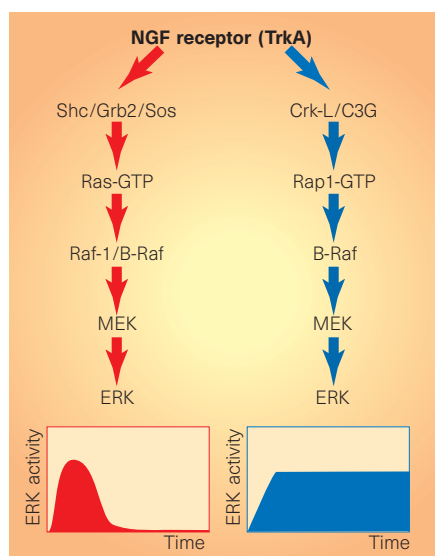


Figure 1 Different pathways activated by nerve growth factor (NGF). York *et al.*¹ have found that sustained activation of extracellular-signal-regulated kinases (ERKs) occurs through a hitherto unknown pathway that involves the small GTPase Rap1 — all pathways were previously thought to act through another small GTPase, Ras. In the Ras pathway, Shc and Grb2 are the adaptor proteins that link Sos — the Ras guanine-nucleotide-exchange factor — to the NGF receptor (TrkA). In the Rap1 pathway, however, the Crk-L protein links C3G, the Rap guanine-nucleotide-exchange factor, to TrkA.

phosphorylates Mek1 or Mek2 (Fig. 1). In mammalian cells, the Meks can also be activated by, among other proteins, A-Raf and B-Raf. York *et al.*¹ present a new twist to the pathway that leads to activation of ERK because they argue that, after NGF stimulation, the early phase of ERK activation is mediated by Ras, but sustained activation of the pathway is due to activation of Rap1. Furthermore, Rap1 is not acting through Raf-1, but through B-Raf. To study the relationship between the initial and sustained phases of activation, the authors expressed a mutant form of Ras that blocks activation of the endogenous protein, and a Rap1 mutant that may act in the same way. They found that the Ras- and Rap-dependent phases of ERK activation seem to be independent.

The idea that Rap1 may be responsible for the sustained activation of ERKs is intriguing. Relatively little has been done to examine the signalling pathways that are responsible for sustained activation of these kinases. Moreover, the compelling evidence that Ras must be activated for the initial phase of ERK activation, together with the fact that, in PC12 cells, NGF produces sustained activation of Ras-GTP whereas EGF produces transient activation of Ras and ERKs⁷, may have led to the false assumption that everything is due to Ras. It would now be nice to know whether EGF also gives a transient activation of Rap1.

Rap1 was discovered through an expression cloning strategy for suppressors of Ras transformation. The mechanism by which Rap1 suppresses oncogenic Ras-driven transformation is still a puzzle — Rap1 could sequester effectors away from Ras, or, alternatively, Rap1 may activate a signalling pathway that suppresses the action of Ras. Rap1 is about 50% similar to Ras in primary sequence and, although it binds Raf-1 with a lower affinity than does Ras⁸, interactions between B-Raf and Rap1 have not been characterized.

A number of issues need to be addressed from the work of York *et al.*¹. The sustained activation of ERK seems to result from activation of Rap1, yet expression of a mutant Rap that blocks sustained ERK activation does not block outgrowth of PC12 neurites. It is clear from previous studies⁵, however, that inhibition of ERK activation does block NGF-stimulated differentiation of PC12. Further inhibition of Ras function by microinjecting a Ras-neutralizing antibody blocks differentiation of PC12 cells² — clearly indicating a role for Ras-dependent signalling.

In other cell systems, Rap1-mediated signalling seems to impair activation of ERKs^{9,10}, and it will be interesting to find out whether the responses of different cells to Rap1 signalling depend on the isoforms of B-Raf that they express. B-Raf was originally thought to be expressed only in neuronal tissue, but different isoforms are now known to be expressed in many cell types¹¹, and B-Raf is essential for embryonic development of blood vessels¹². To complicate interpretation further, at least one isoform of B-Raf can be activated by Ras¹³. But the work of York *et al.* points to new directions for the analysis of Rap1 function, and this, together with the simpler ways¹⁴ for measuring activation of Rap1, suggests that there will be increased interest in these enigmatic proteins. □

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1. York, R. D. *et al.* *Nature* **392**, 622–626 (1998).
2. Hagag, M., Halegoua, S. & Viola, M. *Nature* **319**, 680–682 (1986).
3. Vossler, M. R. *et al.* *Cell* **89**, 73–82 (1997).
4. Traverse, S. *et al.* *Curr. Biol.* **4**, 694–701 (1994).
5. Pang, L., Sawada, T., Decker, S. J. & Saltiel, A. R. *J. Biol. Chem.* **270**, 13585–13588 (1995).
6. Marshall, C. J. *Cell* **80**, 179–185 (1995).
7. Muroya, K., Hattori, S. & Nakamura, S. *Oncogene* **7**, 277–281 (1992).
8. Herrmann, C., Horn, G., Spaargaren, M. & Wittinghofer, A. *J. Biol. Chem.* **271**, 6794–6800 (1996).
9. Cook, S. J., Rubinfeld, B., Albert, I. & McCormick, F. *EMBO J.* **12**, 3475–3485 (1993).
10. Boussiotis, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L. & Nadler, L. M. *Science* **278**, 124–127 (1997).
11. Barnier, J. V., Papin, C., Eychene, A., Lecoq, O. & Calothy, G. *J. Biol. Chem.* **270**, 23381–23389 (1995).
12. Wojnowski, L. *et al.* *Nature Genet.* **16**, 293–297 (1997).
13. Marais, R., Light, Y., Paterson, H. F., Mason, C. S. & Marshall, C. J. *J. Biol. Chem.* **272**, 4378–4383 (1997).
14. Franke, B., Akkerman, J. W. & Bos, J. L. *EMBO J.* **16**, 252–259 (1997).

Daedalus

The eye of mercury

A big telescope mirror never gives a perfect image. Atmospheric fluctuations, and the distortion of the mirror under its own weight as the telescope tracks its stellar target, degrade its focusing. The answer is adaptive optics. An array of fast-acting piezoelectric elements on the back of the mirror can distort it in just the right way to correct the aberrations as they arise. A computer control system, looking at a specific star in the field or a laser beam traversing the optics, drives the elements so as to tweak the mirror continuously into optimum adjustment.

Daedalus is now taking this idea to extremes. A really adaptive mirror would need no optical figuring at all; any random piece of silvered glass could in principle be bent into the perfect shape for the task in hand. Sadly, glass or ceramic cannot be bent very far. But a liquid mirror could be made into any shape whatever, by imposing the right forces on it.

The obvious liquid is mercury. Imagine, says Daedalus, a large dish of mercury with an array of electrodes distributed through it, and a set of coils by which a complex magnetic field can be imposed on it. A current in a magnetic field produces a force, which will appear as hydrostatic pressure in the mercury. So instead of having a flat surface imposed purely by gravity, it will develop some subtle shape determined by the interaction of the field with the currents. Great mathematical cunning would be needed to deform the mercury into a focusing surface, and even greater cunning to superimpose whatever second-order deviations were needed to cancel the effects of atmospheric 'twinkling'; but the thing seems feasible. Such a telescope would need no tilting or steering. Sufficiently strong currents could tilt and shape its mercury surface into just the right form to focus light from any given target onto a fixed CCD retina; and could change its form continuously so as to track that target across the sky.

But this is an ultimate design. Daedalus's pilot scheme lets the mirror gaze vertically upwards, and steers its scrutiny around the sky by means of a big plane mirror or prism above it. These, of course, can be very cheap and crude; the infinitely adaptive optics will correct for their imperfections. Only the chromatic aberration of the prism will defeat it — but even this will be useful. Every object seen by the telescope will be drawn out into a perfect line spectrum.

David Jones

Hesse-Honegger's hand-work

Cornelia Hesse-Honegger is fascinated by the beauty of bugs. After the Chernobyl disaster she set out with her paintbox on the trail of mutated insects, convinced that they were the result of radiation poisoning.

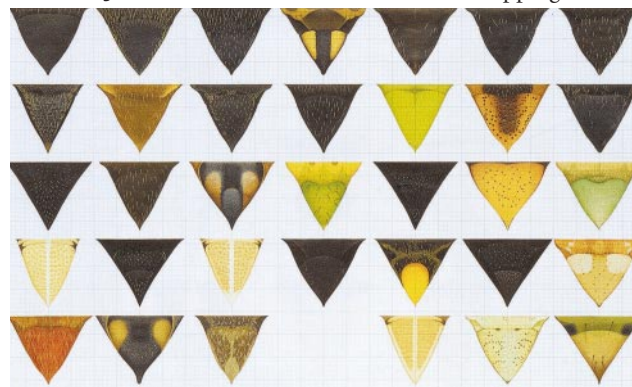
Martin Kemp

The hand-drawn image continues to cling obstinately to its roles in the art of scientific illustration. It is less obviously central now than in the eras when it was the only way to originate representations, but it still does jobs that technologically-generated images cannot. The unique, directed interplay that occurs in the acts of seeing, controlled representation in line, tone and colour, and communication retains a human potency that cannot be equalled by any other means.

Yet is this potency 'subjective' — like a work of art is supposed to be — and therefore 'unscientific'? The charge of subjectivity has been levelled at the amazing watercolours of deformed insects made by the Swiss painter, Cornelia Hesse-Honegger. They may be very striking, and even 'accurate' on their own terms, but can they be 'taken and used in evidence'? Are they, in short, works of art or scientific illustrations?

Hesse-Honegger was trained as a zoological illustrator in Zurich, where she drew for publication, among other specimens, induced mutations in the *Drosophilidae*. After the arrival of her children, she worked increasingly on her own visual agenda, concentrating upon the local population of leaf bugs in their adult (imago) stage "because of their beautiful patterns and colours".

The direction and purpose of her work was redefined in 1986, the year of the Chernobyl disaster. Already aware of the sensitivity of insects as bioindicators of the environment, and unconvinced by reassurances about the lack of danger posed by radiation levels below 5 rem, she studied leaf bug and *Drosophila* populations along the cloud trail in Sweden and Switzerland, and subsequently around Chernobyl itself.



Hesse-Honegger's Leaf Bugs (*Miridae*, several species), part of the wing and scutellum (undeformed), 1979, found in Gockhausen, Switzerland.



Hesse-Honegger's Harlequin Bug (*Pentatomidae*), with deformed scutellum and asymmetrical patches, 1991, found near the Three Mile Island nuclear plant, Pennsylvania.

Visually attuned by her scrupulous earlier depictions of the patterned beauty of such features as the leaf bug's scutellum, she exploits her perceptual and representational skills to draw our eyes into the miniature world of deformations she is uncovering — not only in zones affected by such major incidents but also downwind of normally operating nuclear plants such as Sellafield in north-west England. The legacy of her scientific training is apparent in her systematic mapping and counting of the occurrence of

deformed specimens. At Three Mile Island in Pennsylvania she concluded that "the worst deformations were only located in the E and ESE direction of the plant in an area within one mile of the... plant. No other studies were available."

It is not my intention to offer a judgement on her subsequent disputes with the majority scientific view that what she is recording is statistically insignificant and without correlation to low levels of ionizing radiation, but rather to sustain the right of the obsessional enquirer to probe uncomfortably into visual corners where dark questions lurk. Her probing is highly personal in motivation and aesthetic means.

She speculates that her mutated flies "are perhaps closer to today's reality of nature or, maybe, are prototypes for a future aesthetics of nature". Such aesthetics are subjective in the true sense, but every one of her watercolours carries deep implications for issues of objective judgement. □

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Florida's mystery coral-killer identified

An unusual coral disease appeared on the Florida Reef Tract in June 1995. It was distinct in its microbiology, its pattern of tissue degradation, the species susceptible to it, and its regional distribution. Symptoms included a sharp line between healthy and diseased tissue, as occurs with other coral diseases, but the pathogen responsible for the new outbreak seemed more virulent, affected a wider variety of species, and destroyed tissue much more rapidly than these other 'line' or 'band' diseases. We have identified the pathogen responsible for this new disease as a new species of *Sphingomonas*.

The disease first targeted the elliptical star coral, *Dichocoenia stokesi* Milne-Edwards and Haime. Affected colonies¹ exhibited a sharp line between apparently healthy tissue and a thin zone of bleached tissue grading into exposed coral skeleton (Fig. 1a). Similar lines are associated with other coral diseases², but these colonies of diseased *D. stokesi* exhibited an unusually rapid rate of tissue degradation: up to two centimetres per day. Also, and equally unusually, tissue loss started from the base of the colony.

Within four months of its discovery, the disease had spread about 200 kilometres along the Florida Keys (Fig. 1b). Mortality rates in the *D. stokesi* population, determined from repeated surveys of 20-metre-diameter plots³, averaged 26% over an 11-week period (with a range of 0 to 38% per plot).

Surveys of *D. stokesi* populations on five reefs ($n = 1,196$ colonies) revealed a clumped distribution of infected colonies (Morisita's index of dispersion⁴, $I = 1.84$, $\chi^2 = 49.76$, d.f. = 27, $P < 0.005$; comparison to Poisson distribution, $\chi^2 = 33.1$, d.f. = 4, $P < 0.001$). Overall disease incidence was 20.1% (with a range of 0–33.3% per site). There was a correlation with water depth ($r = 0.42$, d.f. = 24, $P < 0.05$), with the highest incidence of disease at 14 metres (with a range of 1.8–16 m). The number of diseased *D. stokesi* colonies was strongly correlated with the density of all colonies of this species regardless of depth ($r = 0.88$, d.f. = 24, $P < 0.001$), suggesting that the disease is contagious.

In the 1995 outbreak, 16 further species of coral and a hydrocoral were observed with symptoms identical to those of the diseased *D. stokesi*. The other corals affected were *Agaricia agaricites*, *A. lamarcki*, *Colpophyllia natans*, *Dendrogyra cylindrus*, *Diploria labyrinthiformis* (Fig. 1c), *D. strigosa*, *Eusmilia fastigiata*, *Madracis decactis*, *M. mirabilis*, *Manicina areolata*, *Meandrina meandrites*, *Montastrea anularis* (species complex), *M. cavernosa*, *Siderastrea siderea*, *Solenastrea bournoni* and *Stephanocoenia michelinii*, and the hydrocoral *Millepora alcorni*.

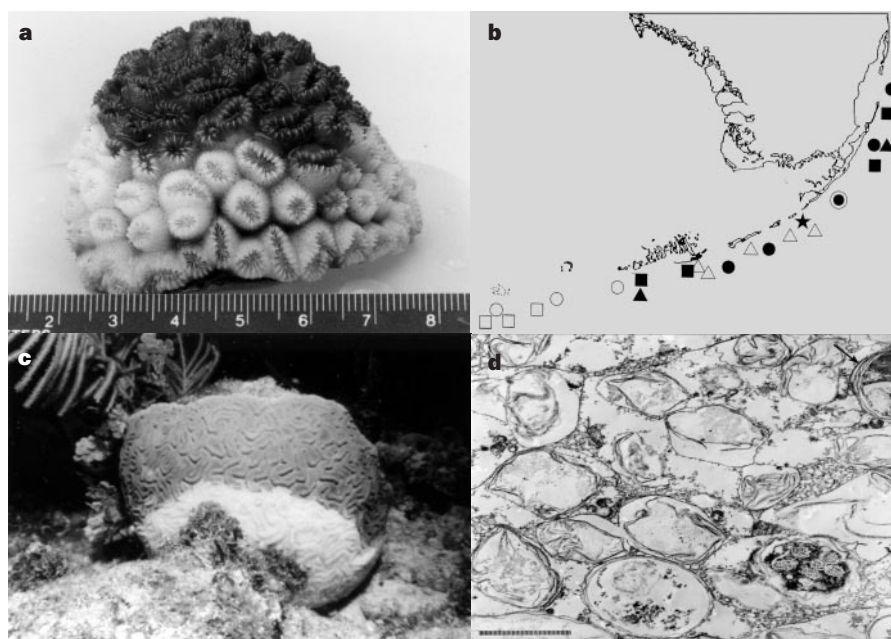


Figure 1 Diseased corals. **a**, Freshly collected diseased colony of *Dichocoenia stokesi* (scale in cm). **b**, Regional survey of diseased *D. stokesi* on reefs of the Florida Reef Tract. Data were collected during three research cruises in August (circles), September (squares) and October (triangles) of 1995. Black symbols, reefs with active disease; white symbols, disease-free reefs; star, site of the first disease observation (7 June 1995, Alligator Reef; K. Nedimyer, personal communication); double circle, site of highest disease incidence (Conch Reef). **c**, *In situ* photograph of diseased *Diploria labyrinthiformis*. **d**, Transmission electron micrograph of gastroderm from the diseased tissue zone of *D. stokesi*. The zooxanthellae have collapsed and degenerated, with multiple periplast membranes (arrow, upper right) and remnants visible within host cells. Methodology has been described¹⁰. Scale bar, 5.0 μm .

No microbial population was visible on the surface of diseased corals. Transmission electron microscopy of the bleached tissue zone revealed degenerated coral tissue with remnants of zooxanthellae, which accounted for the bleached appearance, but no demonstrable pathogens (Fig. 1d).

Samples collected from the surface of the disease line of three infected coral species (*D. stokesi*, *Dendrogyra cylindrus*, and *Diploria labyrinthiformis*) exhibited a predominance of small, motile, Gram-negative rods (measuring 1×1.5 micrometres) at concentrations of 10^5 cells per millilitre. Fewer bacteria were present in samples from apparently healthy tissue and denuded skeletal areas (10^3 and 10^4 cells per millilitre, respectively). Bacterial colonies from diseased tissue were uniform in colour, size and texture, but non-disease-line samples gave rise to mixed bacterial populations.

A bacterial isolate from diseased *D. stokesi* was characterized metabolically by obtaining profiles based on cluster analysis of patterns of carbon-source use⁵ (95 different carbon sources) using Biolog GN plates, and genetically by using the polymerase chain reaction to amplify 16S ribosomal RNA sequences.

The metabolic profiles obtained placed the disease isolate as being most closely relat-

ed to the bacterial genus *Sphingomonas*, a genus first described in 1990 (ref. 6). Genetic analysis placed the isolate in the α -subclass of the *Proteobacteria*, matching most closely the *Erythromicrobium*/*Erythrobacter*/*Sphingomonas* groups (exhibiting an identity of 89, 87 and 86%, respectively). The genera *Erythromicrobium* and *Erythrobacter* (not in the Biolog database) are facultative aerobic photoheterotrophs, but extracts from a culture of the disease isolate incubated under light yielded no photosynthetic pigments. This suggested that the isolate may be a new species of the genus *Sphingomonas*.

Laboratory experiments suggested that the bacterial isolate is the disease pathogen: two healthy colonies of *D. stokesi* placed on marine agar plates inoculated with the isolate became infected at their bases and lost all tissue within three days, whereas control colonies remained healthy. This identification of a single bacterium as pathogen contrasts with the results from microbiological studies of other coral diseases.

Further experiments suggested that the pathogen is readily transmissible: freshly collected, healthy colonies placed in the same aquaria with freshly collected, diseased ones also died.

In the two years following 1995, the reefs

of the Florida Keys were virtually free of disease recurrence. However, in 1996 we recorded new outbreaks in the western portion of the Florida Reef Tract (Key West to the Dry Tortugas), and in 1997 on the reefs north of Miami. In each year (1995–1997) the disease incidences were seasonal, occurring from June to October.

Such repeated, severe infestations may restructure Florida's reef communities, as has occurred as a result of disease in other coral reefs^{7–9}.

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1. Richardson, L. L., Goldberg, W. M., Carlton, R. G. & Halas, J. C. *Rev. Biol. Trop.* (in the press).
2. Peters, E. C. in *Pathobiology of Marine and Estuarine Organisms* (eds Couch, J. A. & Fournie, J. W.) 388–444 (CRC, Boca Raton, 1993).
3. Kuta, K. G. & Richardson, L. L. *Coral Reefs* 15, 219–223 (1996).
4. Morisita, M. *Res. Popul. Ecol.* 4, 1 (1962).
5. Ritchie, K. B. & Smith, G. W. *J. Mar. Biotechnol.* 3, 105–107 (1995).
6. Yabuuchi, E. I. *et al. Microbiol. Immunol.* 32, 99–119 (1990).
7. Lessios, H. A. *Annu. Rev. Ecol. Syst.* 19, 371–393 (1988).
8. Aronson, R. B. & Precht, W. F. *Paleobiology* 23, 326–346 (1997).
9. Smith, G. W., Ives, L. D., Nagelkerken, I. A. & Ritchie, K. B. *Nature* 383, 487 (1996).
10. Goldberg, W. M. & Taylor, G. T. *Mar. Biol.* 125, 655–662 (1996).

Termites fumigate their nests with naphthalene

Termite nests provide a controlled environment and physical defence for the colonies of insects they house¹. Another important factor involved in colony defence may be volatile chemicals present in the nest: we have found the hydrocarbon naphthalene in extracts of the nest material produced by Formosan subterranean termites. This is the first time



Figure 1 Soldier termites following a naphthalene trail. Naphthalene solution (4 μ l) in hexane (2.03 mg ml⁻¹) was streaked on the left-hand pencil circle (final concentration of naphthalene was 0.045 μ M cm⁻¹). The right-hand circle was streaked with 4 μ l hexane as a control.

naphthalene has been found naturally associated with any insect species.

Formosan subterranean termites (*Coptotermes formosanus* Shiraki (Rhino-termidae)) use saliva and excrement to cement soil and masticated wood into a maze of enclosed galleries to make nests^{1,2}. We found between 50.56 and 214.6 micrograms per kilogram of naphthalene in extracts of this nest material, which is known as 'carton'.

Naphthalene acts as a general arthropod fumigant which is used against clothes moths and carpet beetles, a microbial inhibitor and an anthelmintic agent^{3,4}. The chemical is also used as a repellent against mammals and birds: bats, squirrels, rabbits, pigeons, sparrows and starlings³.

The volatility of this compound would allow it to permeate the closed system of the termite nests and shelter tubes⁵. In this system, it may be used for chemical defence against natural enemies such as ants, pathogenic microorganisms and nematodes. One group of these enemies, pathogenic fungi, cause mortalities that limit the establishment of termite colonies in the laboratory⁵. Ants are able to inhibit microbial pathogens, an important attribute that enables them to flourish in the soil habitat⁶. In termite nests, naphthalene as an antiseptic agent, as well as other nest fumigants, may inhibit the growth of such pathogenic microorganisms.

We have shown that naphthalene can inhibit natural fungi from proliferating and also compared the effect of naphthalene on *C. formosanus* and the red imported fire ant, *Solenopsis invicta* Buren, one of the most dominant predators. We found that *C. formosanus* had higher tolerance to naphthalene than did *S. invicta*; ants were paralysed at concentration levels that had no visible effect on termites.

The use of naphthalene as a fumigant by Formosan subterranean termites is unique. However, the function of naphthalene may not be limited to defence. We also discovered that naphthalene elicits

trail-following behaviour in Formosan subterranean termite soldiers (Fig. 1).

Kaib⁷ has argued that, in the adaptive evolution of termite defence strategies, the most important driving force is predation pressure from ants. Predator–prey interactions between ants and termites are constant, unlike occasional or seasonal predation by vertebrates⁷. Ants are social insects with well developed recruitment systems enabling them to use and defend food sources rapidly. These characteristics can create constant pressure on a termite colony. To repel such continued predation pressure, nest fumigation may represent an optimal defence strategy.

Previously, petroleum, coal and the products of incomplete combustion of organic matter were considered to be the only sources of naphthalene in nature⁸. However, the compound has been found as a constituent of *Magnolia* flowers and in the forehead region of male white-tailed deer, *Odocoileus virginianus*^{9,10}. But how termites incorporate naphthalene in their nests and the mechanism of their tolerance to the chemical remain a mystery.

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1. Noirot, C. in *Biology of Termites* Vol. 2 (eds Krishna, K. & Weesner, F. M.) 73–125 (Academic, New York and London, 1970).
2. King, E. G. & Spink, W. T. *Ann. Entomol. Soc. Am.* 62, 536–542 (1969).
3. *Naphthalene Registration Standard* 99 (Environmental Protection Agency, Washington, DC, 1981).

4. Bolton, D. M. & Eaton, L. G. in *The MERCK Index*, 8th edn (eds Stecher, P. G., Windholz, M. & Leahy, D. S.) 713 (MERCK, Rahway, New Jersey, 1968).
5. Higa, S. Y. Flight, colony foundation and development of the gonads of the primary reproductive of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. (Thesis, Univ. Hawaii, 1981).
6. Wilson, E. O. in *The Insect Societies* 27–74 (Belknap, Harvard Univ. Press, Cambridge, Massachusetts, 1971).
7. Kaib, M. in *Chemistry and Biology of Social Insects* (eds Eder, J. & Rembold, H.) 406–407 (Peperny, Munich, 1987).
8. *Naphthalene BUA Report 39* (ed. GDCH Advisory Committee on Existing Chemicals of Environmental Relevance) 11–23 (VCH, New York, 1989).
9. Azuma, H., Toyota, M. A., Asakawa, Y. & Kawanos, S. *Phytochemistry* **42**, 999–1004 (1995).
10. Gassett, J. W. et al. Volatile compounds from the forehead region of male white-tailed deer (*Odocoileus virginianus*). *J. Chem. Ecol.* **23**, 569–578 (1997).

Vitamin C exhibits pro-oxidant properties

Vitamin C is marketed as a dietary supplement, partly because of its 'antioxidant' properties. However, we report here that vitamin C administered as a dietary supplement to healthy humans exhibits a pro-oxidant, as well as an antioxidant, effect *in vivo*.

We conducted a study¹ involving 30 healthy volunteers (16 females and 14 males aged between 17 and 49) whose diets were supplemented with 500 milligrams per day of vitamin C (ascorbic acid) for 6 weeks. We assessed the levels of oxidative damage to peripheral blood lymphocytes in terms of modified DNA bases. The level of 8-oxoguanine was found to decrease on supplementation relative to both placebo (calcium carbonate; 500 mg per day for 6 weeks) and baseline measurements, whereas the level of 8-oxoadenine increased.

For each volunteer, blood was collected at 3-weekly intervals for up to 12 weeks (6 weeks on placebo, 6 weeks on vitamin C)

and then another sample was taken 7 weeks after completion of the vitamin C course (washout period). In each case, levels of plasma ascorbate were determined, lymphocytes were isolated and their DNA extracted before analysing oxidative damage.

Supplementation of diets with 500 mg per day of vitamin C resulted in a significant increase in ascorbate levels in the plasma (about 60%) compared with both pre-supplementation and placebo (data not shown). Following the washout period, vitamin C levels returned to the concentrations observed at baseline and in the placebo. In contrast, there was no change in ascorbate concentrations during oral placebo treatment in comparison with the baseline value.

We used gas chromatography–mass spectrometry (GC–MS)^{2–4} to assess the levels of 8-oxoguanine and 8-oxoadenine, which are markers for DNA damage mediated by oxygen radicals. Supplementation of diets with 500 mg per day of vitamin C resulted in a significant decrease ($P < 0.01$ by ANOVA compared with both baseline and placebo) in 8-oxoguanine levels (Fig. 1). No significant differences from baseline damage to DNA bases was observed during the placebo treatment. Furthermore, following the 7-week 'washout' period, levels of 8-oxoguanine returned to those observed at baseline and during placebo treatment.

In contrast, supplementation with vitamin C resulted in a significant increase ($P < 0.01$ by ANOVA compared with baseline and with placebo values) in 8-oxoadenine levels in DNA isolated from lymphocytes. Again, there were no significant differences during the placebo treatment. During the washout, the mean level of 8-oxoadenine returned to that observed at baseline or during placebo, such that there was a statistically significant decrease

in 8-oxoadenine compared with the vitamin C supplementation period ($P < 0.01$). There were no changes in either lymphocytes or neutrophil counts during either placebo or vitamin C treatments (data not shown), confirming that the alterations noted in 8-oxopurine levels in lymphocyte DNA were not due to gross changes in cell type.

We established baseline values for the 8-oxoguanine and 8-oxoadenine lesions in human lymphocyte DNA. Mean values obtained for 8-oxoguanine (30 lesions per 10^5 guanine bases) and 8-oxoadenine (8 lesions per 10^5 adenine bases) were remarkably similar to those found in other *in vivo* systems^{5–7}. These results re-emphasize that GC–MS is a powerful technique for measuring oxidative DNA damage as it can simultaneously quantify more than one modified DNA base.

Endogenous oxygen radicals are capable of damaging cellular biomolecules such as DNA⁸. Most of these species produced *in vivo* are quenched by antioxidant defences. However, a fine balance exists that may be disrupted in favour of oxidants (oxidative stress), giving rise to an accumulation of biomolecular damage, which in turn may play a role in major diseases such as cancer, rheumatoid arthritis and atherosclerosis⁹. It has been postulated that dietary intervention with vitamin C may reduce oxidative stress and thereby prevent such diseases.

Although the antioxidant nature *in vivo* of vitamin C has been questioned¹⁰, it is nonetheless marketed as supplements in doses of 500 mg or more per day as an 'antioxidant'. Our discovery of an increase in a potentially mutagenic lesion, 8-oxoadenine^{11,12}, following a typical vitamin C supplementation should therefore be of some concern, although at doses of less than 500 mg per day the antioxidant effect may predominate.

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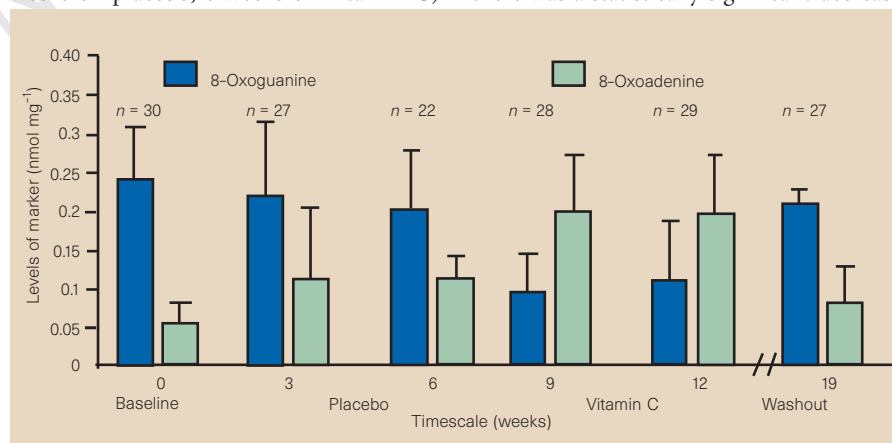


Figure 1 Levels of 8-oxoguanine and 8-oxoadenine in lymphocyte DNA from healthy subjects during vitamin C supplementation as measured by gas chromatography–mass spectrometry. Values represent the mean plus 1 s.d. Data were analysed by general linear model analysis of variance (ANOVA), with subsequent comparison between means using either Tukey one-way ANOVA, or Fisher's least significant difference test if there was a significant subject effect. One nanomol of 8-oxoguanine (8-oxoadenine) per mg DNA is equivalent to about 124 8-oxoguanines (8-oxoadenines) per 10^6 guanines (adenines).

1. Antioxidant Programme (Ministry of Agriculture, Fisheries and Food, UK, 1995).
2. Dizdaroğlu, M. *Meth. Enzymol.* **234**, 3–16 (1994).
3. Hamberg, M. & Zhang, L.-Y. *Analyt. Biochem.* **229**, 336–344 (1995).
4. Ravanat, J.-L., Turesky, R. J., Gremaud, E., Trudel, L. J. & Stadler, R. H. *Chem. Res. Toxicol.* **8**, 1039–1045 (1995).
5. Liu, P. K. et al. *J. Neurosci.* **16**, 6795–6806 (1996).
6. Olinski, R. et al. *Cancer Lett.* **106**, 207–215 (1996).
7. Jaruga, P. & Dizdaroğlu, M. *Nucleic Acids Res.* **24**, 1389–1394 (1996).
8. Aruoma, O. I. & Halliwell, B. *Chemistry in Britain* **27**, 149–152 (1991).
9. Lunec, J. *J. Int. Fed. Clin. Chem.* **4**, 58–63 (1992).
10. Halliwell, B. *Free Rad. Res.* **25**, 439–454 (1996).
11. Kamiya, H. et al. *Nucleic Acids Res.* **23**, 2893–2899 (1995).
12. Wood, M. L., Esteve, A., Morningstar, M. L., Kuziemko, G. M. & Essigmann, J. M. *Nucleic Acids Res.* **20**, 6023–6032 (1992).

Regulatory factor linked to late-onset diabetes?

Maintenance of glucose balance in mammals depends on the production of insulin by the β -cells of the pancreas, in response to raised concentrations of blood glucose. In humans suffering from non-insulin-dependent diabetes (NIDDM), β -cell failure follows chronic resistance to insulin-stimulated glucose uptake and causes the development of hyperglycaemia¹. NIDDM shows a polygenic inheritance pattern in most cases²: defined genetic defects that have little effect on their own, in combination induce diabetes by epistatic interactions³. Here we show that mice heterozygous for the gene *pdx-1*, which encodes a transcription factor for the insulin gene and regulates pancreatic development, have impaired glucose tolerance. This pancreatic nuclear regulatory factor is required for glucose homeostasis even when the pancreas is morphologically normal.

The *pdx-1*-encoded homeodomain protein in mammals (STF-1, IPF-1, IDX-1)⁴⁻⁶ was isolated as a transcriptional regulator of insulin and somatostatin⁷⁻⁹. The protein was first detected in the embryonic pancreatic and duodenal endoderm. But in the pancreas, *pdx-1* expression becomes progressively restricted to islets, where it is produced in more than 90% of β -cells, and in substantially fewer δ -cells (15%) and α -cells (3%)^{10,11}.

Mice that are heterozygous (+/-) for *pdx-1* develop normally, but in *pdx-1* homozygotes (-/-), the branching outgrowth of the pancreas that usually occurs is arrested at an early stage^{11,12}. The relevance of these findings is underscored by a description of a human phenotype lacking a pancreas and associated with a mutation in the *pdx-1* gene^{13,14}. Maturity-onset diabetes occurred in humans heterozygous for this mutation¹³; this prompted us to examine whether *pdx-1* is important for glucose homeostasis in an adult mouse model.

We fasted *pdx-1* wild-type and +/- mice for 14–16 hours and injected them intraperitoneally with 20% glucose (2 grams per kilogram body weight). The blood glucose levels of the wild type underwent a threefold increase within 15 minutes but returned to baseline two hours later (Fig. 1a). By contrast, +/- mice showed a 7–10-tenfold increase in blood glucose levels after 30 minutes. These mice remained hyperglycaemic even after 2 hours (Fig. 1a).

The levels of plasma insulin following glucose administration (not shown) did

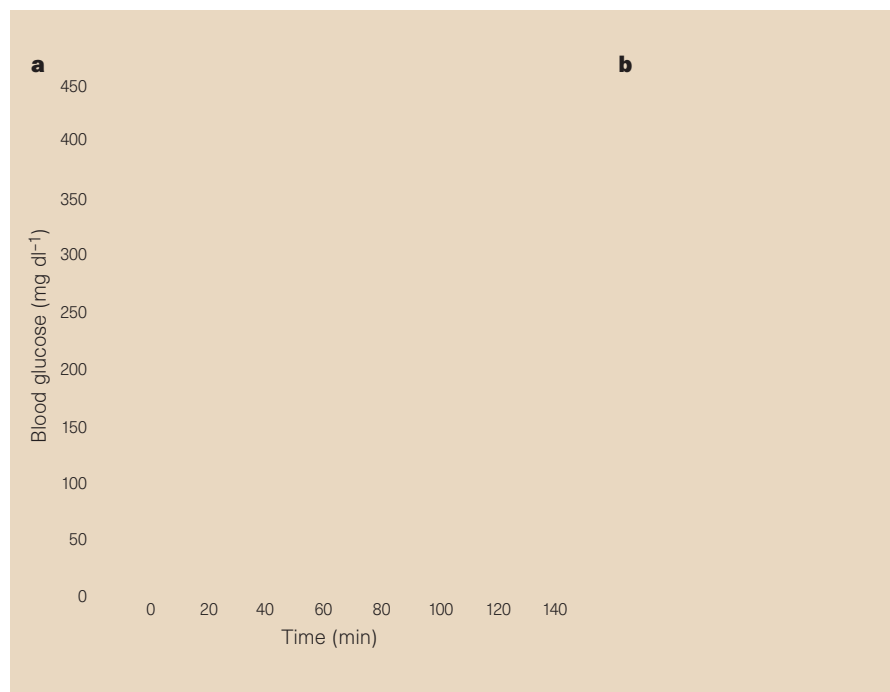


Figure 1 Comparison of wild-type and *pdx-1*-deficient mice. **a**, Glucose tolerance tests on black Swiss mice that are either heterozygous for *pdx-1* (+/-, red) or wild type (green). Mean blood glucose levels over time are shown with standard error ($n = 8$ for wild-type mice, $n = 11$ for +/- mice). Animals were fasted for 14–16 hours before testing. Blood glucose levels were monitored with a glucometer. **b**, Representative islets from 15-week-old wild-type (top) and +/- (bottom) mice. Islets were immunostained with combined antibodies raised against glucagon, somatostatin and pancreatic polypeptide to show the mantle of islet non- β -cells around the core of unstained β -cells. Magnification bar, 50 μ m.

not differ appreciably between wild-type and +/- animals, but these insulin levels were inappropriately low for the degree of hyperglycaemia observed in the +/- mice.

In histological evaluation, the pancreatic islets from the +/- mice appeared somewhat smaller, with a thicker mantle devoid of β -cells, than those of the wild type (Fig. 1b). The mass of β -cells was reduced, but to an extent that was not significant (wild-type mice: 2.80 milligrams $\pm$ 0.44; $n = 8$; +/- mice: 1.76 milligrams $\pm$ 0.13; $n = 4$). However, the mass of non- β -cells was almost doubled in +/- compared with wild-type mice (wild-type mice: 0.47 mg $\pm$ 0.06; +/- mice: 0.82 mg $\pm$ 0.10). This suggests that a deficiency in the *pdx-1* gene may skew the islet cell lineages towards developing into non- β cells.

Our results support the idea that, as well as acting as a regulatory protein for pancreatic development, the protein encoded by *pdx-1* is required for glucose homeostasis in the adult pancreas. Thus a deficiency in *pdx-1* may predispose certain individuals to the development of late-onset diabetes, particularly in the context of other genetic mutations within the insulin-signalling cascade.

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1. Ciaraldi, T. P., Abrams, L., Nikoulina, S., Mudaliar, S. & Henry, R. R. *J. Clin. Invest.* 96, 2820–2827 (1995).
2. Sacks, D. B. & McDonald, J. M. *Am. J. Clin. Pathol.* 105, 149–156 (1996).
3. Bruning, J. et al. *Cell* 88, 561–572 (1997).
4. Leonard, J. et al. *Mol. Endocrinol.* 7, 1275–1283 (1993).
5. Miller, C. P., McGhee, R. E. Jr & Habener, J. F. *EMBO J.* 13, 1145–1156 (1994).
6. Ohlsson, H., Karlsson, K. & Edlund, T. *EMBO J.* 12, 4251–4259 (1993).
7. Peers, B., Leonard, J., Sharma, S., Teitelman, G. & Montminy, M. R. *Mol. Endocrinol.* 8, 1798–1806 (1994).
8. Peers, B., Sharma, S., Johnson, T., Kamps, M. & Montminy, M. *Mol. Cell. Biol.* 15, 7091–7097 (1995).
9. Serup, P. et al. *Biochem. J.* 310, 997–1003 (1995).
10. Guz, Y. et al. *Development* 121, 11–18 (1995).
11. Offield, M. F. et al. *Development* 122, 983–995 (1996).
12. Jonsson, J., Carlsson, L., Edlund, T. & Edlund, H. *Nature* 371, 606–609 (1994).
13. Stoffers, D. A., Zinkin, N. T., Stanojevic, V., Clarke, W. L. & Habener, J. F. *Nature Genet.* 15, 106–110 (1997).
14. Stoffers, D. A., Ferrer, J., Clarke, W. J. & Habener, J. F. *Nature Genet.* 17, 138–139 (1997).

correction

In the Scientific Correspondence article entitled "Inhibition of ICE slows ALS in mice" by R. M. Friedlander, R. H. Brown, V. Gagliardini, J. Wang and J. Yuan (*Nature* 388, 31; 1997), the mouse strain used was G93A (rather than G93R as published).

New testament on retroviruses

Retroviruses

edited by John M. Coffin, Stephen H. Hughes and Harold E. Varmus
Cold Spring Harbor Laboratory Press: 1998.
Pp. 843. \$180

Robin A. Weiss

Teaching AIDS on an immunology course recently, I posed the question: how did retroviruses acquire their name? One wit volunteered the answer: "Because they were discovered back in the 1970s." In the HIV era, retroviruses are so pervasive that their replication by reverse transcription appears commonplace, with no sense of the revolution caused by the discovery of reverse transcriptase in 1970. Indeed, it often comes as a surprise to students who have not yet studied virology to learn that other RNA viruses, such as the agents causing influenza or poliomyelitis, do not make DNA. It is the RNA-dependent RNA polymerases that are now regarded as curiosities.

Yet where can one go to bone up on retroviruses? For several years there has been no general text that encompasses up-to-date knowledge of retroviruses. So this is the book we have been waiting for, and no-one should be disappointed.

As the editors state in the preface to *Retroviruses*, the forerunner was published 15 years ago. But this is not a new edition; rather it is a new testament, written entirely anew and with a different, appropriately modern, structure. The old testament had its genesis 25 years ago when Cold Spring Harbor Laboratory published a textbook called *The Molecular Biology of Tumor Viruses*. It was superseded in 1980 and 1982 by two separately edited texts on *DNA Tumor Viruses* and *RNA Tumor Viruses* which became the bibles of molecular virologists working in or entering the field. The titles betray the focus of effort in those days, for HIV made its debut only in the 1985 paperback edition of *RNA Tumor Viruses* which had to be expanded to a second volume to include the new discoveries as well as a compendium of retrovirus genome maps. New maps and sequence data are provided in the present book by two excellent appendices with references to Web sites for international databanks.

Retroviruses begins fittingly with a homage to Howard M. Temin by David Baltimore. There is a scholarly, historical introductory chapter by Peter K. Vogt illustrating the several paradigm shifts that occurred with retroviruses, and citing Thomas Kuhn to justify the volume: "Textbooks are produced in the aftermath of scientific revolutions". This is followed by six synoptic gospels on retroviral organization and replication. A short 'intermezzo' or

epistle by the editors on the interactions of retroviruses and their hosts introduces further apostolic chapters on endogenous genomes, vectors, pathogenesis, the apocalypse of HIV, and the revelations of immunological and therapeutic approaches to the control of retroviral infections. The only aspects that are somewhat superficially covered are transmission (other than HIV) and natural immune responses. Although frequently mentioned, the natural history of retroviruses receives less coherent treatment than molecular virology.

Retroviruses will be read in most detail by scientists, although it is a useful volume for physicians and veterinarians, too. This is a serious reference book to be cherished by specialist and novice alike. Its weighty volume (2.5 kilograms) is addressed to the research community rather than to the undergraduate. As the editors state, the chapters are not intended to be read and digested strictly in order, but according to the interest of the reader. Each starts with a concise summary and is handsomely illustrated. Chapters are individually referenced and cover retroviruses in a comparative manner, drawing on common features and principles as well as on the fascinating variety of retroviral pathogenesis. The index, with a

few exceptions (such as HIV neuropathogenesis), provides a genuinely useful homing device to the immense breadth and depth of the subject.

The retrovirologist will continually dip into the volume for reference. Graduate students will now have an easier time writing their theses. The nonviral molecular and cell biologist will find many nuggets of useful information and exposition. My copy disappeared from my office the day it arrived. Better order two copies at once. □

Robin A. Weiss is at the Institute of Cancer Research, Fulham Road, London SW3 6JB, UK.

Connoisseur's collection

Made to Measure: New Materials for the 21st Century

by Philip Ball
Princeton University Press: 1998. Pp. 458.
\$29.95, £18.95

A. Lindsay Greer

The English are not prominent as wine growers, yet this in no way conflicts with their being able connoisseurs — indeed it

Reproductive thermodynamics

We may think we know the answer to the question 'what is sex?' but Lynn Margulis and Dorion Sagan offer a new philosophical slant on the subject. Their book *What is Sex?* (Simon and Schuster, \$37.50) views sex simply as a consequence of thermodynamics. Organisms are open systems with orifices through which both energy and matter can flow. Photosynthesis involves flow of gases through stomata (right) and, in the same manner, sex is merely a flow of matter and dissipation of energy. Sex exists, they argue, purely because the organisms that reproduce sexually have survived; it has no inherent advantage over asexual reproduction.



facilitates an unbiased appreciation of what the world has to offer. In such a manner, Philip Ball — not a materials scientist but an associate editor of *Nature* — presents a guide to materials for the twenty-first century. The book opens, though, in eighth-century Samarkand with the extraction, from the Chinese, of the secrets of paper-making. Ball points out that, in the post-medieval information revolution, the role of the material paper tends to be underestimated.

Such spicing with historical background is a hallmark of this book. It is refreshing to find a book on 'new' materials that sets them in their context, recognizing that they have a pedigree, and exploiting a description of predecessors to set off the distinguishing features of the new.

The range of materials and applications considered is vast — from artificial blood to artificial legs, light-emitting silicon to holographic memories, photostrictive titanates to microporous clays, hydrogels for drug delivery to designed bacterial polymers, fuel cells to artificial diamonds. Occasionally, Ball may stray too far — few would treat gene therapy within the range of new materials.

Omissions are mostly intended. High-temperature superconductors are avoided for the good reasons that they have had extensive publicity already and are now the subject, not of breakthroughs but of hard grind to make their use practicable. Ball is aware that new, expensive materials are destined for specialized uses; justifiably, he chooses not to focus on structural materials. All the same, within his theme of materials 'made to measure', it is a shame not to recount the story of modern steels designed in the virtual mill of the computer. The coverage, strong on history, is also well up to date. It is good to find, for example, a thorough treatment of the new blue light-emitting diodes.

The book is far from being just a list of diverse materials. It also offers analysis of trends. Ball's picking out of themes in biomimetics, for example, is masterly. In this way the book should have much more than ephemeral interest. Ball is ready to acknowledge the wider factors influencing the adoption or rejection of a new material — industrial realities, wider economics, ecological concerns. Many books on new materials have more hyperbole than substance; this is not one of them. Ball writes of his materials with enthusiasm, but tempered by realism. He recognizes that most (but far from all) new materials will be passing wonders.

For a book on this subject, it is ironic that its material presentation — paper, binding, printing, illustrations — is old-fashioned. This may make it less immediately attractive to buy than its accessible writing deserves. The background science (band structure of semiconductors, architecture of cells) necessary to appreciate the main message is

Mathematics of the natural world



As Philip Ball explains in *Made to Measure*, reviewed left, mollusc shells not only are sophisticated composite materials but can be ornately decorated with coloured pigments produced by cells in the proten outer mantle. The patterns are frozen 'chemical waves' generated by complex, nonlinear interactions between cells during shell growth, which modulate the production of pigment. The images here are taken from the enlarged version of Hans Meinhardt's *The Algorithmic Beauty of Sea Shells* (Springer, DM89, \$54.95, £34), the first edition of which was reviewed in *Nature* 375, 745 (1995).

described with a fluency and accuracy that puts most practising scientists to shame.

Inevitably there are small errors. Some may be distressed by Ball's sometimes uncertain distinction between strength and stiffness, others by his description of the Mancunian James Joule as a Scot. Yet the treatment overall is authoritative, and is supported by a good bibliography.

There are some 160 references to individuals or institutions involved with current developments. Of these, 61% are from the United States, 11% from the United Kingdom (equal to all of continental Europe combined), and 10% from Japan. Does this reflect the true distribution of progress, or rather national variations in countries' ability and willingness to promote their wares? Whatever the answer, Ball's selected case of materials is worth having. □

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When language doesn't add up

Twice As Less: Black English and the Performance of Black Students in Mathematics and Science

by Eleanor Wilson Orr

Norton: 1997. Pp. 242. \$12.95, £9.95 (pbk)

Geoffrey K. Pullum

In 1972, Washington DC's Board of Education drew lots to pick 41 students from the city's public schools for free enrolment at the private, coeducational Hawthorne School. Nearly all were native speakers of African American Vernacular English (AAVE). The teachers were at first baffled by the new students' curious though systematic conceptual errors in mathematics, but ultimately developed strategies that improved results dramatically.

Fifteen years later, Eleanor Wilson Orr published this book about Hawthorne's experiment. The 1997 reissue has a new introduction commenting on the Oakland 'Ebonics' furore (see *Nature* **386**, 321; 1997). Orr's thesis is that black children's mathematical misapprehensions are often due to contrasts between AAVE and standard English, particularly regarding grammatical function words (prepositions, comparative 'than', equative 'as').

The topic merits attention. Starting out with a different prepositional system might well interfere with acquisition of arithmetical concepts expressed in English through metaphorically extended preposition meanings: a quarter of 16 is obtained by dividing 16 by 4 or dividing 4 into 16 or dividing 16 into quarters, and so on. And there are indeed some prepositional use differences between AAVE and standard English. Orr offers thought-provoking transcriptions of student reasoning; and some of what they reveal might be traceable to translation problems. (This possibility was the grain of truth in the policy of acknowledging AAVE for which the Oakland school board was so mercilessly mocked.)

But Orr fails to establish AAVE's causal role. Superficial and anecdotal examples of AAVE are presented along with data from a different language, Guyanese Creole. No clear linguistic aetiologies for mathematical conceptualization difficulties emerge. For example, the attempt to link AAVE's negative concord rule to confusion of 'half' with 'twice' (equating 'half as small' with 'twice as small') is intriguing, but the proffered speculations about a determinative influence are less than convincing.

Some of the cited misunderstandings seem clearly nonlinguistic. For example,

there is evidence of students thinking that to show why some abstract relationship holds one need only give an example in which it holds. We need not take AAVE to be implicated in such signs of unfamiliarity with abstract thinking. It is common today to find speakers of standard American English, even at college level, who reason similarly. □

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Detecting the undetectable

Cosmic Bullets

by Roger Clay and Bruce Dawson
Addison-Wesley/Allen and Unwin: 1998.
\$22 (hbk), £7.99 (pbk)

Peter L. Biermann

The most energetic particles we measure in the Universe come from outer space and hit the Earth at a rate of only about one particle per square kilometre per century. And yet we have now measured about a dozen such particles, with several detector systems, and their energy is near 10^{21} eV.

Roger Clay and Bruce Dawson have written a small book describing how these particles were discovered, how we became certain that their energy is really that high, and how the search for their origin and nature is heating up. The book nicely provides the setting of the discovery and explains why in theory such particles should not really be detectable: because they interact with the microwave background, there should be only negligible flux beyond about 5×10^{19} eV. The exciting finding is that no cut-off has yet

been seen in the sparse data, with the highest energy events having values near 2×10^{20} eV and 3×10^{20} eV.

The authors trace the excitement of the research community in 1996, even telling the story of the possible correlation of the particles with the supergalactic plane. This plane is the locus in the sky where most cosmologically nearby galaxies and radio galaxies are, so one might expect that the high-energy particles can be traced to at least this distribution. Sadly, the authors just missed the events of May 1997, when the brightest gamma-ray burst so far was seen at gamma, X-ray, optical and radio wavelengths. Gamma-ray bursts may or may not be related to the highest energy particles, but they are surely good candidates to investigate.

As described in the book, the idea with the longest staying power in the whole story would seem to be that these particles arise from acceleration in gigantic shockwaves in powerful radio galaxies. As we have studied radio galaxies for many decades it is surprising that we have not confirmed or refuted such a concept and moved on to the more challenging possibilities such as tracing the high-energy particles to topological defects created in the Big Bang or — perhaps even more exciting — tracing them to supersymmetric partners of normal particles.

A book of this type must, of necessity, make compromises in elucidating the physics. But I think that some of its short-cuts are a little odd. For example, of Max Planck's discovery, the authors write: "he developed a mathematical technique for explaining the spectra of heated bodies". The reader needs to have a good knowledge of the history of science to understand what they are referring to.

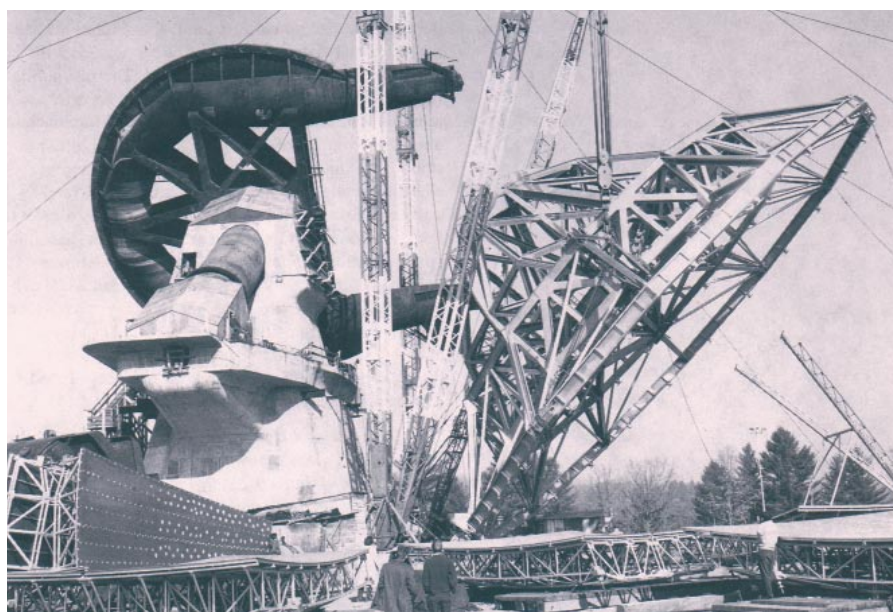
Also, although the book conveys well the excitement of some of the steps in the stony path of discovery, it seems that the delight of detecting something new is virtually impossible to capture in mere dry words. (I speak as one of the participants in the story.) On the whole, however, the book is eminently readable for both experts and laypeople, particularly a young readership.

The basic nature of matter is commonly explored in expensive large accelerators such as those at Stanford, Fermilab and the European Laboratory for Particle Physics. The high-energy events now observed through air-showers are far beyond any energy obtainable in these machines on the ground. The book culminates in describing the Auger project, the ambitious plan to erect two gigantic air-shower arrays in Earth's northern and southern hemispheres. These air-shower arrays will be sufficiently large for us to be able to observe many high-energy events, and so eventually do physics at energies of 10^{21} eV and possibly higher. □

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Fell to Earth: the Chicago Air Shower Array is aimed at detecting high-energy cosmic and gamma rays.



Sky eye: raising the superstructure of the 140-ft telescope at Green Bank in the 1960s.

History repeats itself

The History of Radio Astronomy and the National Radio Astronomy Observatory

by Benjamin K. Malphrus

Krieger: 1996. Pp. 210. \$49.50, £44.50

Leslie Sage

As a long-time user of telescopes at the US National Radio Astronomy Observatory (NRAO), I was eager to read this book to learn more about my 'scientific origins'. I was very disappointed by it; the discussion seems shallow as both science and history.

The emphasis is on the development of the NRAO site at Green Bank, West Virginia, along with some historical background. Most of the text is about the (now collapsed) 300-ft telescope and the recently closed 140-ft telescope, but there is a very brief chapter containing sections about the 36-ft telescope at Kitt Peak in Arizona, the Very Large Array in New Mexico, and the new Green Bank Telescope, which is the replacement for the 300-ft antenna. The Millimetre-Wave Array is not mentioned at all.

Although the author discusses extensively the scientific results obtained from the older telescopes, he does so in a way so laden with jargon that only an astronomer could understand them. Moreover, he does not explain why the results are (or at least were) interesting in terms of advancing our physical understanding of the Universe. Rather, the discussion is about phenomenology, so that even optical astronomers may sometimes be left in the dark about why the observations were made.

I was struck by the lack of historical analysis. There are parallels between the building of the 140-ft telescope and the new Green

Bank Telescope, which has run into similar delays in construction. I feel that the author is doing a disservice to the astronomical community by not exploring the underlying issues, although I imagine that there are some who would prefer not to have such problems aired in public. As George Santayana said: "Those who cannot remember the past are condemned to repeat it."

The story of NRAO, and its companion organization at optical wavelengths (the National Optical Astronomy Observatories), is one that deserves to be told to a wide audience, as the two have revolutionized how astronomy is done, not just in the United States, but throughout the world. By giving telescope time based on the scientific merit of the ideas, rather than the institutional affiliation of the observer, they have led to the field exploding over the past 40 years, and to a revolution in our knowledge of the Universe. I wish this book had done a better job of telling that story. □

Leslie Sage is an assistant editor of *Nature*.

corrections

● In I. J. Good's review of *The Conscious Universe* by Dean Radin (*Nature* 389, 806; 1997), an editorial addition led to an inaccurate statement being attributed to the author rather than to the sceptical psychologist Mark Hansel. The fourth sentence in the penultimate paragraph should have read: "Radin quotes Hansel as saying that three *P* values, each of 0.01, amount to one of 10^{-6} , and that he (Hansel) would find that convincing."

● Some of the bibliographical details for *Embryology: Constructing the Organism* edited by Scott F. Gilbert and Anne M. Raunio (*Nature* 391, 857; 1998) were given incorrectly. The book has 513 pages, not 240, and is published in hardback only.

New in paperback

The Killing of the Countryside

by Graham Harvey

Vintage, £7.99

Looks at the current state of farming in Britain, and makes the case for a sustainable future.

The Complete Fables of Aesop

Translated by Olivia and Robert Temple, with an introduction by Robert Temple

Penguin, £5.99, \$8.95

First English translation ever to make available the complete corpus of 358 fables. The stories provide fascinating glimpses of ordinary life in ancient Greece, and include satirical tales of alien creatures — apes, camels, lions and elephants — which presumably originated in Libya and Egypt.

The Conscious Mind: In Search of a Fundamental Theory

by David J. Chalmers

Oxford University Press, £9.99

"Chalmers's dualism is a very mild one and does not invoke any spooky soul-stuff", C. Koch, *Nature* 381, 123 (1996).

The Machinery of Life

by David S. Goodsell

Copernicus, \$19.50

"The drawings are among the most instructive one can find in structural biology, and the mechanisms of life are elegantly explained.... The genius of the book is in its simplicity.... Important insights are stated succinctly and boldly.... The most elegant mechanisms are presented in an easily accessible but consistently informative way", H. P. Erickson, *Nature* 365, 306 (1993).

Darwin's Dreampond: Drama in Lake Victoria

by Tijs Goldschmidt

MIT Press, \$15

"Goldschmidt was part of the Dutch haplochromine ecology survey team that devoted more than 20 years to collecting baseline ecological, morphological, taxonomic and fisheries data on the cichlids, which provide the basic protein source for the people around Lake Victoria.... [His] book is both unusual and eminently readable. He alternates between personal narrative... and scientific passages", Axel Meyer, *Nature* 383, 590 (1996).

Hal's Legacy: 2007's Computer as Dream and Reality

edited by David G. Stork, with a foreword by Arthur C. Clarke

MIT Press, \$17.50

Brings together science and pop culture. Contributions by various scientists include essays on supercomputer design with regard to speech synthesis, common-sense reasoning, emotions, lip reading and chess playing.

Chemokines and leukocyte traffic

Marco Baggiolini

Over the past ten years, numerous chemokines have been identified as attractants of different types of blood leukocytes to sites of infection and inflammation. They are produced locally in the tissues and act on leukocytes through selective receptors. Chemokines are now known to also function as regulatory molecules in leukocyte maturation, traffic and homing of lymphocytes, and the development of lymphoid tissues.

Last century's pathologists knew that leukocytes emigrate from the blood across the wall of microvessels and accumulate in inflamed tissues. The purpose of this migration, a process called diapedesis, remained unknown until Elias Metschnikoff¹ showed that leukocytes engulf and kill bacteria and recognized diapedesis as a fundamental mechanism of host defence. Today, ten years after the discovery of interleukin (IL)-8, chemokines are seen as the stimuli that largely control leukocyte migration. Chemokines have been in the limelight since 1996, when it was discovered that some of their receptors function as binding sites for AIDS viruses. Obviously, sharing receptors with human immunodeficiency virus (HIV) is not the *raison d'être* of chemokines. Their main business is attracting leukocytes².

The basics

Despite its large size, the chemokine family is remarkably homogeneous, and the properties originally ascribed to IL-8 (ref. 3) are still generally valid for the 40 or so human chemokines that have been described so far. Chemokines are small proteins with four conserved cysteines forming two essential disulphide bonds (Cys1–Cys3 and Cys2–Cys4). CXC and CC chemokines are distinguished according to the position of the first two cysteines, which are either adjacent (CC) or separated by one amino acid (CXC). Chemokines have a short amino-terminal domain preceding the first cysteine, a backbone made of β -strands and the connecting loops found between the second and fourth cysteines, and a carboxy-terminal α -helix of 20–30 amino acids. The backbone has a well ordered structure whereas the N- and C-terminal domains are disordered, especially at their extremities⁴. A protein with two instead of four conserved cysteines, lymphotactin⁵, and a chemokine-like structure with three amino acids between the first two cysteines (CX₃C motif) at the N-terminal end of a mucin structure^{6,7} have also been described.

The effects of chemokines on leukocytes are mediated by heptahelical receptors coupled to GTP-binding proteins. The most impressive effect is the shape change that is observed within seconds after addition of an attractant to a leukocyte suspension, as shown in Fig. 1 for neutrophils. Polymerization and breakdown of actin leads to the formation and retraction of lamellipodia, which function like arms and legs of the migrating cells. Stimulation also induces the upregulation and activation of integrins, which enable the leukocytes to adhere to the endothelial cells of the vessel wall before migrating into the tissues⁸. Several other rapid and transient responses are characteristic of the activation of leukocytes by chemokines, such as the rise in the intracellular free calcium concentration, the production of microbicidal oxygen radicals and bioactive lipids, and the release of the contents of the cytoplasmic storage granules, such as proteases from neutrophils and monocytes, histamine from basophils and cytotoxic proteins from eosinophils^{2,9}.

Most chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes^{2,10}. Some chemokines seem to fulfil housekeeping functions, however. They may be involved in

leukocyte maturation in the bone marrow, the traffic and homing of lymphocytes, and the mechanisms that ensure the renewal of circulating leukocytes.

Chemokine–receptor interactions and antagonists

Five receptors for CXC chemokines and eight receptors for CC chemokines have been characterized¹¹. As shown in Table 1, most receptors recognize more than one chemokine, and several chemokines bind to more than one receptor, indicating that redundancy and versatility are characteristic for the chemokine system (Fig. 2). CXC- and CC-chemokine receptors (CXCRs and CCRs) only recognize chemokines of the corresponding subfamily. Chemokines have two main sites of interaction with their receptors, one in the N-terminal region and the other within an exposed loop of the backbone that extends between the second and the third cysteine¹². The N-terminal binding site is essential for triggering of the receptor. It is believed that the receptor recognizes the loop region first, and that this interaction is necessary for the correct presentation of the triggering domain¹². Analogues that still bind effectively but do not signal and thus act as receptor antagonists were obtained by amino-acid deletion or modification of the N-terminal region of IL-8 and related CXC chemokines², N-terminal truncation of MCP-1, MCP-3 and RANTES^{2,13}, or N-terminal elongation of RANTES¹⁴ and MCP-3 (ref. 15). Potent CXCR4 antagonists were obtained by modification of the first two N-terminal residues of SDF-1 (ref. 16). Antagonists^{2,9} and receptor-blocking antibodies¹⁷ inhibit chemokine-induced responses. The search for antagonists has been boosted by the discovery that chemokine receptors, together with the T-cell differentiation antigen CD4, act as recognition sites for HIV-1 (ref. 18). Chemokines and the HIV surface protein gp120 recognize overlapping receptor epitopes and compete for binding. HIV infection is prevented by chemokines^{19–21}, but also by chemokine-derived antagonists^{14,22}, and similar effects can be obtained by

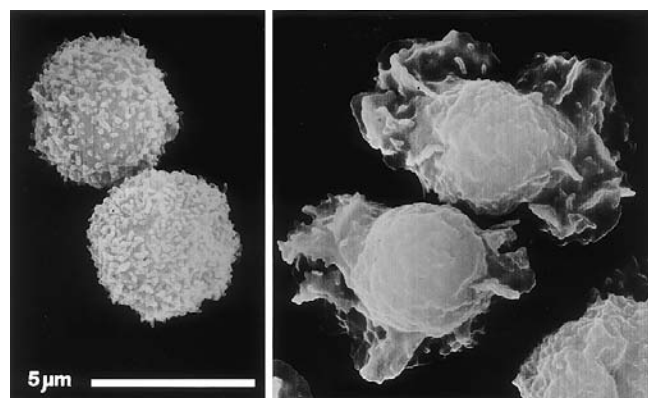


Figure 1 Shape change of human neutrophil leukocytes. Cells in buffered saline are shown by scanning electron microscopy before (left) and 5 seconds after (right) stimulation with a chemoattractant.

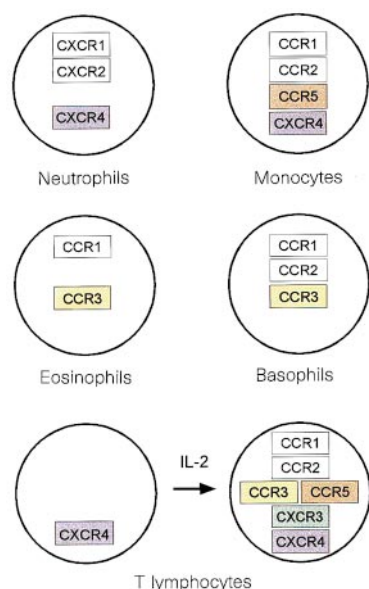


Figure 2 Main chemokine receptors expressed on human leukocytes. The scheme shows four CXC- and five CC-chemokine receptors that have been extensively characterized in terms of function and binding properties. The SDF-1 receptor, CXCR4, is widely expressed and CCR3 occurs in eosinophils, basophils and (a subset of) T lymphocytes. By far the greatest variety of receptors is seen in T lymphocytes in which expression depends on the state of cell activation. CCR4, CCR6 and CCR7, which are also found in T lymphocytes, are omitted as conditions for their expression are still being investigated.

chemokine-unrelated, low-molecular-weight substances²³. These findings indicate that the efforts under the AIDS flag may eventually yield antagonists to be used for broader therapeutic applications, for example, in chronic inflammation, autoimmunity, allergic diseases and prevention of transplant rejection.

The rationale for anti-inflammatory therapy based on interference with the chemokine system has been established in animal models²⁴, as illustrated here by a few examples. Lung reperfusion injury²⁵ and urate-crystal-induced arthritis²⁶ in rabbits showed regression after treatment with an anti-IL-8 antibody. Anti-inflammatory effects were also obtained in rat glomerulonephritis with antibodies against MIP-2 (ref. 27), in cutaneous delayed hypersensitivity with antibodies against MCP-1 (ref. 28) and in mouse allergic airway inflammation with antibodies against MIP-1 α and RANTES²⁹. In view of the redundancy among chemokines, neutralization at the receptor level may be more promising, and first encouraging results have been obtained with CC-chemokine antagonists in murine models of arthritis. Administration of an MCP-1 antagonist prevented the onset of arthritis in the MRL-*lpr* mouse and reduced the symptoms once the disease had developed³⁰, and the antagonist MetRANTES significantly inhibited collagen-induced arthritis in DBA/1 mice³¹.

Allergic inflammation

The observation that RANTES and MCP-3 activate eosinophil and basophil leukocytes, inducing chemotaxis and the release of histamine and leukotrienes, was the first hint that chemokines are involved in allergy³². Then eotaxin was discovered, a powerful attractant of eosinophils that immediately gained attention for its potential function in asthma and other forms of allergic inflammation. Eotaxin is expressed in the lungs of animals with asthma and in human tissues where eosinophils accumulate⁹. The eotaxin receptor, CCR3, is present not only in eosinophils but also in basophils^{33,34} and a subset of T lymphocytes with T_H2 helper properties^{35,36}. Whereas basophils and eosinophils release mediators that induce

Table 1 Human chemokine receptors and their ligands*

Receptor	Chemokine
CXCR1	IL-8, GCP-2
CXCR2	IL-8, GRO α / β / γ , NAP-2, ENA78, GCP-2
CXCR3	IP10, Mig
CXCR4	SDF-1
CXCR5	BCA-1/BLC
CCR1	RANTES, MIP-1 α , MCP-2, MCP-3
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	Eotaxin, eotaxin-2, RANTES, MCP-2, MCP-3, MCP-4
CCR4†	TARC, RANTES, MIP-1 α , MCP-1
CCR5	RANTES, MIP-1 α , MIP-1 β
CCR6	LARC/MIP-3 α /exodus
CCR7	ELC/MIP-3 β
CCR8	I-309 $\ddagger$
CX ₃ CR1	Fraktalkine/Neurotactin

* The receptors for the lymphocyte-specific chemokines SLC/6CKine/exodus-2 and DC-CK1/PARK are unknown.

† There is disagreement about the selectivity of CCR4, which was first described as a receptor for RANTES, MIP-1 α and MCP-1 and was later shown to be specific for TARC³⁸.

‡ See references 68, 69.

smooth muscle contraction, vascular permeability, mucus secretion, and airway hyperreactivity, T lymphocytes contribute to inflammation by producing cytokines such as IL-4, which enhances IgE production, and IL-5, which primes and activates eosinophils and basophils. It is remarkable that the pathophysiologically relevant leukocytes share CCR3 and can be recruited concomitantly to sites of allergic inflammation by the same chemokines. T lymphocytes expressing CCR3 are found with eosinophils in atopic dermatitis, nasal polyps and ulcerative colitis, whereas the lymphocytes in non-allergic infiltrates are generally CCR3-negative³⁶. As shown in Table 1, CCR3 binds several chemokines. Eotaxin and the recently identified eotaxin-2 (ref. 37) bind only to CCR3 whereas RANTES, MCP-2, MCP-3 and MCP-4 also bind to other CC-chemokine receptors⁹. In mice with a deletion of the eotaxin gene, allergen-induced eosinophil infiltration into the lungs is retarded, but the defect is compensated at later stages by other chemokines³⁸. A more sustained effect should result from disruption of the gene encoding CCR3.

T-lymphocyte recruitment

Although lymphocytes were known to accumulate at sites of immune and inflammatory reactions, attractants that induce these responses have been identified only recently. RANTES, MIP-1 α and MIP-1 β (ref. 2) were the first chemokines for which lymphocyte-chemotactic activity was reported. The monocyte-chemotactic proteins (MCP-1, -2, -3 and -4) are also potent attractants of T lymphocytes, natural killer and dendritic cells⁹. Chemokine-receptor expression varies considerably in lymphocytes. CCR1, CCR2 (ref. 39) and CCR5 (ref. 40) are upregulated by IL-2, whereas other stimulatory conditions, such as exposure to anti-CD3 and/or anti-CD28 antibodies, downregulate receptors and chemotaxis. These observations indicate that T lymphocytes may migrate in response to chemokines after IL-2-mediated proliferation, but not during antigen-dependent activation. Upregulation of chemokine receptors in lymphocytes may enhance susceptibility to HIV infection^{40,41}. Unlike many CC chemokines, which attract monocytes, basophils and eosinophils in addition to T lymphocytes, the CXC chemokines IP10 and Mig are selective for IL-2-activated T lymphocytes. They recognize only one receptor, CXCR3, which is restricted to T lymphocytes⁴². Production of IP10 and Mig is induced by interferon- γ (IFN- γ), which inhibits the expression of most other chemokines², and this property is a further element of selectivity. In viral infections or delayed-type hypersensitivity reactions, IP10 and Mig are locally upregulated by IFN- γ and available for the recruitment of effector lymphocytes.

Two types of CD4-positive helper T lymphocytes, T_H1 and T_H2, are distinguished according to the cytokines they produce⁴³. As the accumulation of lymphocytes belonging to one or the other subset

influences the course of the local immune response, it was interesting to study the mechanism of recruitment. T_H1 and T_H2 cells differ in chemokine-receptor expression and their responsiveness to chemokines^{35,36,44–46}. CCR5 is expressed preferentially in T_H1 cells whereas CCR3 and CCR4 seem to be characteristic of T_H2 cells. It can be predicted that chemokine receptors will soon be used as markers for subpopulations of helper T lymphocytes.

Homeostatic functions in lymphoid tissues

During their development and differentiation, T and B lymphocytes move through different tissue compartments. Although the paths, the role of adhesion molecules in the recognition of homing sites, and several highly effective chemokines are known, it is still difficult to understand how this intricate cellular trafficking is regulated⁴⁷. Some pieces of the puzzle, however, are in place. Particularly interesting is a group of newly identified CC chemokines, including TARC, ELC, SLC, LARC and DC-CK1 (see Table 1 for synonyms), which, except for LARC, are expressed constitutively at high levels in the thymus, lymph nodes and other lymphoid tissues. They all attract T lymphocytes and most of them also attract B lymphocytes, which bear selective receptors, namely CCR4 for TARC, CCR6 for LARC, and CCR7 for ELC⁴⁸. DK-CK1 is produced by dendritic cells of germinal centres and T-lymphocyte areas of secondary lymphoid organs and is chemotactic for naive T lymphocytes, suggesting a role in the initiation of an immune response⁴⁹. TECK is produced by thymic dendritic cells and is chemotactic for murine macrophages, dendritic cells and thymocytes⁵⁰. The restricted, constitutive production of these chemokines in lymphoid tissues and their apparent selectivity for receptors expressed by lymphocytes suggest that they are involved in the regulation of physiological lymphocyte traffic.

An example of such a housekeeping function of chemokines comes from observations in mice lacking BLR1, a putative chemokine receptor that is highly expressed in B lymphocytes⁵¹. Disruption of the BLR1 gene leads to a loss of inguinal lymph nodes and a defective formation of primary follicles and germinal centres in the spleen and Peyer's patches. Receptor-deficient B lymphocytes enter the T-cell areas of these tissues, but fail to migrate into B-cell areas. These results suggested the existence of chemokines that direct lymphocyte homing into specific anatomical areas and regulate the development of functional lymphoid tissues. The ligand for BLR1 (now called CXCR5) is a novel CXC chemokine, BCA-1/BLC, that is expressed in lymphoid tissues and is selective for B lymphocytes^{52,53}.

A chemokine with potential involvement in lymphocyte maturation and other homeostatic functions is SDF-1, which was originally described as a growth factor for B-lymphocyte precursors⁵⁴. Murine SDF-1 attracts resting T lymphocytes *in vitro* and *in vivo* with unusually high efficacy⁵⁵. Human SDF-1, which differs from its murine homologue by a single residue (Ile instead of Val at position 18) is chemotactic for T lymphocytes, monocytes and neutrophils^{20,21}, which all express CXCR4 (Fig. 2). The gene encoding CXCR4 was cloned in several laboratories⁹ and CXCR4 was later found to act as a co-receptor for T-lymphocyte-tropic HIV-1 strains^{18,56}. Mice lacking the SDF-1 gene have severely impaired lymphopoiesis and abnormally low numbers of B-lymphocyte and myeloid bone-marrow precursors⁵⁷. SDF-1 is chemotactic for pro- and pre-B cells, which depend on stromal-cell contact for growth and differentiation, but not for more mature forms⁵⁸. SDF-1 was also shown to induce chemotaxis of CD34-positive cells of different lineages⁵⁹. All these results indicate that SDF-1 may attract progenitor B cells into the microenvironment of stromal cells where growth and differentiation factors are released⁵⁸. More generally, SDF-1 may be involved in directing progenitor cells into the appropriate maturation sites in the bone marrow⁵⁹ and may support the colonization of the bone marrow by haematopoietic precursors during embryogenesis⁵⁵. In addition, the finding that mice lacking

SDF-1 have a defective ventricular septum of the heart⁵⁷ suggests that SDF-1 may not only act on leukocytes and their precursors. A role in morphogenesis is conceivable because SDF-1, unlike most chemokines, is expressed constitutively in several tissues, and its receptor, CXCR4, is found in leukocytes but also in different types of tissue cells⁹.

Future directions

The progress in the chemokine field has been rapid but largely straightforward, and most chemokine actions that have been reported are related to leukocyte migration and recruitment. A major surprise, of course, was the observation that some chemokines block HIV infection⁹, which initiated an unprecedented burst of research activity in most leading laboratories in the chemokine and AIDS fields and led to the discovery of chemokine receptors as recognition sites for viral infection¹⁸. By comparison, other unforeseen developments were less spectacular and have not attracted as much attention and research power. The observation that the Duffy blood group antigen, the recognition site for infection by the malarial parasite *Plasmodium vivax*, binds chemokines was unexpected. DARC (Duffy antigen receptor for chemokines) is a heptahelical receptor that is expressed on erythrocytes, endothelial cells of postcapillary venules and the Purkinje cells of the cerebellum. It binds several chemokines of the CXC and CC subfamilies, but does not signal and its function in this context remains uncertain⁶⁰. Major functions that may not depend on leukocyte migration have been attributed to chemokines, namely the inhibition and stimulation of blood-vessel formation (angiogenesis)⁶¹ and leukocyte maturation in the bone marrow (myelopoiesis)^{62,63}. The mechanisms of these effects and the chemokine receptors involved, however, are still being studied.

Where can major advances be expected? One answer that immediately comes to mind is 'lymphocytes'. In view of the identification of several novel chemokine receptors in lymphoid tissues and of lymphocyte-selective chemokines, I am convinced that most migration responses in the complicated trafficking of lymphocytes of different types and degrees of activation will eventually turn out to be mediated by chemokines. Research in this field will open therapeutic opportunities for autoimmune diseases, transplantation and immune deficiencies. Another interesting, therapeutically oriented area is that of chemokine antagonists. Finding antagonists is at present a major goal of many biotechnology and pharmaceutical companies. The prospects are exciting because it has been shown that antagonists are effective, and some low-molecular-weight compounds that block chemokine receptors are already at hand²³. In addition, new insights into the regulation of leukocyte traffic may be gained by the search for novel chemokines and chemokine receptors with restricted tissue expression. Deletion of chemokine or chemokine-receptor genes, which was very informative in the case of MIP-1 α (ref. 62), SDF-1 (ref. 57) and BLR1 (ref. 51), is also a promising approach.

Chemokine activities can also be modulated by interfering with signal transduction, another major area for future research. Most studies so far have used neutrophils or cells transfected with receptors for IL-8, C5a or fMet-Leu-Phe, which signal in similar ways^{2,9}. Upon IL-8 binding, receptor coupling with a *Bordetella pertussis* toxin-sensitive G protein, usually of G α_{12} type, initiates the signalling cascade leading to activation of a phosphatidylinositol-specific phospholipase C, protein kinase C, small GTPases, Src-related tyrosine kinases, phosphatidylinositol-3-OH kinases and protein kinase B^{2,9,64}. Phospholipase C delivers two second messengers, inositol-1,4,5-trisphosphate, which releases Ca²⁺ from intracellular stores leading to a transient rise of the cytosolic Ca²⁺ concentration, and diacylglycerol, which activates protein kinase C. Mobilization of Ca²⁺ is essential for granule release and superoxide production, but is not required for the cytoskeletal rearrangements leading to shape change². Phosphatidylinositol-3-OH kinases

can be activated by the $\beta\gamma$ subunit of G proteins, small GTPases or Src-related tyrosine kinases⁹. Small GTPases regulate cytoskeletal rearrangements involved in adhesion and chemotaxis^{65,66}, mediate activation of phospholipase D and are involved in the assembly of the superoxide-forming oxidase⁶⁶. Signalling by other chemokine receptors has not been studied in comparable depth, and virtually no information is available about receptors that mediate homeostatic rather than inflammatory functions of chemokines, for which different signal-transduction mechanisms can be assumed.

A more speculative issue is the possible role of chemokines in bringing or keeping together cells that form a functional unit. This type of attraction has analogy with chemotaxis and has been suggested for the interaction between stromal cells and progenitor B cells⁵⁸, and between dendritic cells and naive T lymphocytes⁴⁹. Progress here may be made by concentrating on chemokines that are constitutively expressed in various tissues, such as SDF-1 and HCC-1 (ref. 9), and on chemokine receptors expressed in tissue cells, for example, CXCR4 and receptors found in nerve tissues⁶⁷. *In situ* attraction is only a step away from morphogenesis where cells that form a tissue may initially be kept in close contact by attractants. As mentioned, a potential (direct or indirect) role of chemokines in morphogenesis is suggested by the observation that deletion of the SDF-1 gene led to a defective heart formation⁵⁷. □

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1. Metchnikoff, E. *L'immunité dans les Maladies Infectieuses* (Masson & Cie, Paris, 1901).
2. Baggiolini, M., Dewald, B. & Moser, B. Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Adv. Immunol.* **55**, 97–179 (1994).
3. Baggiolini, M., Walz, A. & Kunkel, S. L. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* **84**, 1045–1049 (1989).
4. Rajarathnam, K., Clark-Lewis, I. & Sykes, B. D. ¹H NMR solution structure of an active monomeric interleukin-8. *Biochemistry* **34**, 12983–12990 (1995).
5. Kennedy, J. *et al.* Molecular cloning and functional characterization of human lymphotactin. *J. Immunol.* **155**, 203–209 (1995).
6. Bazan, J. F. *et al.* A new class of membrane-bound chemokine with a CX₃C motif. *Nature* **385**, 640–644 (1997).
7. Pan, Y. *et al.* Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387**, 611–617 (1997).
8. Springer, T. A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301–314 (1994).
9. Baggiolini, M., Dewald, B. & Moser, B. Human chemokines: an update. *Annu. Rev. Immunol.* **15**, 675–705 (1997).
10. Furie, M. B. & Randolph, G. J. Chemokines and tissue injury. *Am. J. Pathol.* **146**, 1287–1301 (1995).
11. Murphy, P. M. Chemokine receptors: structure, function and role in microbial pathogenesis. *Cytokine Growth Fact. Rev.* **7**, 47–64 (1996).
12. Clark-Lewis, I. *et al.* Structure-activity relationships of chemokines. *J. Leukocyte Biol.* **57**, 703–711 (1995).
13. Zhang, Y. J., Rutledge, B. J. & Rollins, B. J. Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. Identification of a mutated protein that inhibits MCP-1-mediated monocyte chemotaxis. *J. Biol. Chem.* **269**, 15918–15924 (1994).
14. Simmons, G. *et al.* Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276**, 276–279 (1997).
15. Masure, S., Paemen, L., Proost, P., Van Damme, J. & Opdenakker, G. Expression of a human mutant monocyte chemotactic protein 3 in *Pichia pastoris* and characterization as an MCP-3 receptor antagonist. *J. Interferon Cytokine Res.* **15**, 955–963 (1995).
16. Crump, M. P. *et al.* Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* **16**, 6996–7007 (1997).
17. Heath, H. *et al.* Chemokine receptor usage by human eosinophils—the importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest.* **99**, 178–184 (1997).
18. D'Souza, M. P. & Harden, V. A. Chemokines and HIV-1 second receptors—confluence of two fields generates optimism in AIDS research. *Nature Med.* **2**, 1293–1300 (1996).
19. Cocchi, F. *et al.* Identification of RANTES, MIP-1 α , and MIP-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**, 1811–1815 (1995).
20. Oberlin, E. *et al.* The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833–835 (1996).
21. Bleul, C. C. *et al.* The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* **382**, 829–833 (1996).
22. Arenzana-Seisdedos, F. *et al.* HIV blocked by chemokine antagonist. *Nature* **383**, 400 (1996).
23. Baggiolini, M. & Moser, B. Blocking chemokine receptors. *J. Exp. Med.* **186**, 1189–1191 (1997).
24. Strieter, R. M. *et al.* "The good, the bad, and the ugly": the role of chemokines in models of human disease. *J. Immunol.* **156**, 3583–3586 (1996).
25. Sekido, N. *et al.* Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. *Nature* **365**, 654–657 (1993).
26. Nishimura, A. *et al.* Attenuation of monosodium urate crystal-induced arthritis in rabbits by a neutralizing antibody against interleukin-8. *J. Leukocyte Biol.* **62**, 444–449 (1997).
27. Feng, L., Xia, Y., Yoshimura, T. & Wilson, C. B. Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody. *J. Clin. Invest.* **95**, 1009–1017 (1995).
28. Rand, M. L., Warren, J. S., Mansour, M. K., Newman, W. & Ringler, D. J. Inhibition of T cell recruitment and cutaneous delayed-type hypersensitivity-induced inflammation with antibodies to

- monocyte chemoattractant protein-1. *Am. J. Pathol.* **148**, 855–864 (1996).
29. Lukacs, N. W. *et al.* Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J. Immunol.* **158**, 4398–4404 (1997).
30. Gong, J. H., Ratkay, L. G., Waterfield, J. D. & Clark-Lewis, I. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-*lpr* mouse model. *J. Exp. Med.* **186**, 131–137 (1997).
31. Plater-Zyberk, C., Hoogwerf, A. J., Proudfoot, A. E. L., Power, C. A. & Wells, T. N. C. Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunol. Lett.* **57**, 117–120 (1997).
32. Baggiolini, M. & Dahinden, C. A. CC chemokines in allergic inflammation. *Immunol. Today* **15**, 127–133 (1994).
33. Ugucioni, M. *et al.* High expression of the chemokine receptor CCR3 in human blood basophils—role in activation by eotaxin, MCP-4, and other chemokines. *J. Clin. Invest.* **100**, 1137–1143 (1997).
34. Yamada, H. *et al.* Eotaxin is a potent chemotaxin for human basophils. *Biochem. Biophys. Res. Commun.* **231**, 365–368 (1997).
35. Sallusto, F., Mackay, C. R. & Lanzavecchia, A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* **277**, 2005–2007 (1997).
36. Gerber, B. O. *et al.* Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. *Curr. Biol.* **7**, 836–843 (1997).
37. Forssmann, U. *et al.* Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. *J. Exp. Med.* **185**, 2171–2176 (1997).
38. Rothenberg, M. E., MacLean, J. A., Pearlman, E., Luster, A. D. & Leder, P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* **185**, 785–790 (1997).
39. Loetscher, P., Seitz, M., Baggiolini, M. & Moser, B. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* **184**, 569–577 (1996).
40. Bleul, C. C., Wu, L. J., Hoxie, J. A., Springer, T. A. & Mackay, C. R. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc. Natl Acad. Sci. USA* **94**, 1925–1930 (1997).
41. Moore, J. P. & Koup, R. A. Chemoattractants attract HIV researchers. *J. Exp. Med.* **184**, 311–313 (1996).
42. Loetscher, M. *et al.* Chemokine receptor specific for IP10 and Mig: structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* **184**, 963–969 (1996).
43. Abbas, A. K., Murphy, K. M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793 (1996).
44. Loetscher, P. *et al.* CCR5 is characteristic for Th1 lymphocytes. *Nature* **391**, 344–345 (1998).
45. Bonocchi, R. *et al.* Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* **187**, 129–134 (1998).
46. Sallusto, F., Lenig, D., Mackay, C. R. & Lanzavecchia, A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and T helper 2 lymphocytes. *J. Exp. Med.* (in the press).
47. Butcher, E. C. & Picker, L. J. Lymphocyte homing and homeostasis. *Science* **272**, 60–66 (1996).
48. Yoshie, O., Imai, T. & Nomiya, H. Novel lymphocyte-specific CC chemokines and their receptors. *J. Leukocyte Biol.* **62**, 634–644 (1997).
49. Adema, G. J. *et al.* A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* **387**, 713–717 (1997).
50. Vicari, A. P. *et al.* TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity* **7**, 291–301 (1997).
51. Förster, R. *et al.* A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* **87**, 1037–1047 (1996).
52. Legler, D. F. *et al.* BCA-1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* **187**, 655–660 (1998).
53. Gunn, M. D. *et al.* A B-cell-homing chemokine made in lymphoid follicles activated Burkitt's lymphoma receptor-1. *Nature* **391**, 799–803 (1998).
54. Nagasawa, T., Kikutani, H. & Kishimoto, T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc. Natl Acad. Sci. USA* **91**, 2305–2309 (1994).
55. Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A. & Springer, T. A. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J. Exp. Med.* **184**, 1101–1109 (1996).
56. Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877 (1996).
57. Nagasawa, T. *et al.* Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**, 635–638 (1996).
58. D'Apuzzo, M. *et al.* The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur. J. Immunol.* **27**, 1788–1793 (1997).
59. Aiuti, A., Webb, J. J., Bleul, C., Springer, T. & Gutierrez-Ramos, J. C. The chemokine SDF-1 is a chemoattractant for human CD34⁺ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34⁺ progenitors to peripheral blood. *J. Exp. Med.* **185**, 111–120 (1997).
60. Hadley, T. J. & Peiper, S. C. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. *Blood* **89**, 3077–3091 (1997).
61. Arenberg, D. A. *et al.* The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. *J. Leukocyte Biol.* **62**, 554–562 (1997).
62. Cook, D. N. The role of MIP-1 α in inflammation and hematopoiesis. *J. Leukocyte Biol.* **59**, 61–66 (1996).
63. Verfaillie, C. M. Chemokines as inhibitors of hematopoietic progenitors. *J. Lab. Clin. Med.* **127**, 148–150 (1996).
64. Tilton, B., Andjelkovic, M., Didichenko, S. A., Hemmings, B. A. & Thelen, M. G-protein coupled receptors and Fcg-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J. Biol. Chem.* **272**, 28096–28101 (1997).
65. Laudanna, C., Campbell, J. J. & Butcher, E. C. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. *Science* **271**, 981–983 (1996).
66. Bokoch, G. M. Chemoattractant signaling and leukocyte activation. *Blood* **86**, 1649–1660 (1995).
67. Horuk, R. S. *et al.* Expression of chemokine receptors by subsets of neurons in the central nervous system. *J. Immunol.* **158**, 2882–2890 (1997).
68. Roos, R. S. *et al.* Identification of CCR8, the receptor for the human CC chemokine I-309. *J. Biol. Chem.* **272**, 17251–17254 (1997).
69. Tiffany, H. L. *et al.* Identification of CCR8: a human monocyte and thymus receptor for the CC chemokine I-309. *J. Exp. Med.* **186**, 165–170 (1997).

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Essential role of mouse telomerase in highly proliferative organs

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We have investigated the role of the enzyme telomerase in highly proliferative organs in successive generations of mice lacking telomerase RNA. Late-generation animals exhibited defective spermatogenesis, with increased programmed cell death (apoptosis) and decreased proliferation in the testis. The proliferative capacity of haematopoietic cells in the bone marrow and spleen was also compromised. These progressively adverse effects coincided with substantial erosion of telomeres (the termini of eukaryotic chromosomes) and fusion and loss of chromosomes. These findings indicate an essential role for telomerase, and hence telomeres, in the maintenance of genomic integrity and in the long-term viability of high-renewal organ systems.

Telomeres are guanine-rich, simple repeat sequences that constitute the physical termini of eukaryotic chromosomes¹. Early work^{2,3} indicated that these terminal structures may be important in chromosome function⁴. Synthesis and maintenance of telomeric repeats are mediated by a specialized ribonucleoprotein complex known as telomerase^{5,6}. The telomerase holoenzyme consists of an essential RNA template^{6,7} and protein components, including one with similarity to reverse transcriptases^{8,13}.

In the absence of telomerase, the failure of DNA polymerase to fully synthesize DNA termini leads to chromosomal shortening with each round of replication. As most somatic human tissues and primary cells possess low or undetectable telomerase activity, continual organ renewal *in vivo* or passage of cells in culture leads to a steady decline in telomere length^{14,15}. Such a reduction in telomere length may play a role in replicative senescence, as constitutive telomerase activity can extend the proliferative capacity of normal human cells¹⁶. Viral oncoproteins or mutational events can drive senescent cultures through additional rounds of replication with continued telomere loss until 'crisis' ensues—an event highlighted by marked genetic instability and cell death. From this crisis period, immortal cells emerge that have reactivated telomerase and maintain stable telomere structures^{17–19}.

The reactivation of telomerase during cellular immortalization matches well with what occurs during tumour progression in mice and humans. In particular, a marked upregulation of telomerase activity occurs in >90% of malignant samples compared with normal adjacent tissue¹⁹. These data led to the speculation that telomerase may be required for the efficient growth of neoplastic cells and that telomerase inhibition could represent a new therapeutic approach for cancer^{14,15}. We have recently shown that primary mouse cells lacking the mouse telomerase RNA (*mTR*) gene, and thus lacking telomerase activity, can still spontaneously become immortalized and generate a fully transformed tumorigenic phenotype after viral oncogenesis²⁰, indicating that telomerase activity may not be limiting for tumorigenesis in the mouse.

The idea that telomeres play an essential role in normal cellular growth and survival has been substantiated in unicellular eukaryotes. In yeast, deletion of the telomerase RNA gene leads to telomere

shortening and, after a generational lag period, to loss of cell viability^{21,22}. The lag period presumably comprises cell divisions during which telomeres shorten before they reach a critical length that is the threshold for chromosome instability and cell death. Elimination of a telomeric end from a yeast chromosome causes a RAD9-mediated cell-cycle arrest and chromosomal loss, indicating that telomeres may function to help cells distinguish intact chromosomes from broken DNA molecules and to maintain genomic stability^{23,24}.

In mammals, an *in vivo* role for telomerase and telomeres in normal cellular growth and long-term maintenance of organ systems has not yet been examined. On the basis of the phenotype of telomerase-null yeast and the great increase in chromosomal aberrations and aneuploidy in *mTR*^{−/−} cells^{20–22}, we expected that telomerase deficiency would have its greatest impact on highly proliferative organ systems. Here we demonstrate progressive adverse effects of telomerase deficiency on the reproductive and haematopoietic systems. These effects on cell renewal, coupled with telomere-length reductions and abnormal cytogenetic profiles, establish a role for telomerase in normal cellular homeostasis at the biological and genomic levels.

Early-generation telomerase-deficient mice

We reported previously a decline in telomere length with each successive generation of the *mTR*^{−/−} mouse²⁰. Here we performed a phenotypic analysis of each generation, and paid particular attention to organ systems with high proliferative activity. Studies of an ageing population of the first-generation (*G*₁) *mTR*^{−/−} mice (from heterozygous intercrosses) did not reveal adverse effects on the overall clinical condition (lifespan, motor behaviour/activity and weight gain) through 20 months of life (data not shown). Similarly, the haematopoietic organ histology and peripheral white and red blood cell counts, as well as the capacity of haematopoietic progenitor cells to grow and differentiate *in vitro* and of mature immunocytes to respond to mitogenic or infectious stimuli, were not significantly different from those of controls (see below). In addition, other organs with a more moderate level of renewal activity, such as intestine and skin, exhibited normal morphology and rates of proliferation and apoptosis (data not shown). These results indicate that telomerase activity *per se* is not essential in organogenesis or postnatal organ function or maintenance. It is possible that stressful conditions leading to increased cell turnover

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(for example, phlebotomy, chronic infection, skin wounding, petactomy) might be able to elicit an age-dependent phenotype in old $mTR^{-/-}$ mice. Preliminary analysis indicates that some G_1 $mTR^{-/-}$ animals in two independent colonies exhibit erosive dermatitis, which might indicate a defect in dermal stem cells (H.W.L. and R.A.D., and E. H. Herrera, J. M. C. Caballero and M.A.B., unpublished observations). At present the limited sample size does not allow us to assign this phenotype unambiguously to the mTR deficiency, although studies are continuing.

On the basis of previous studies in lower organisms that show a temporal lag between telomere loss and diminished cell viability, we suspected that substantial telomere loss would be required before a compromised clinical condition became apparent. Thus, as described²⁰, we have produced additional generations (G_2 , G_3 , G_4 , G_5 and G_6) of $mTR^{-/-}$ mice from successive matings of homozygous-

null intercrosses. Avoidance of inbreeding in the later generations was achieved through regular cousin-mating schemes²⁵ of offspring produced from 20 different G_1 $mTR^{-/-}$ matings; these G_1 mating pairs were in turn derived from 10 different $mTR^{+/-}$ intercrosses.

Reproductive system

Reproductive function is highly dependent on proper germ-cell expansion and development. All matings proved to be productive up to the fifth generation. However, a statistically significant decline in litter size became apparent in the G_4 intercrosses (Fig. 1a; G_4 $P = 0.021$ compared with G_1). No offspring were produced from eight different G_6 intercrosses. Both sexes appear to be affected, as only one in eight G_6 males and one in eight G_6 females produced a litter when mated to a wild-type partner.

Male reproductive system. As the number of spermatocyte

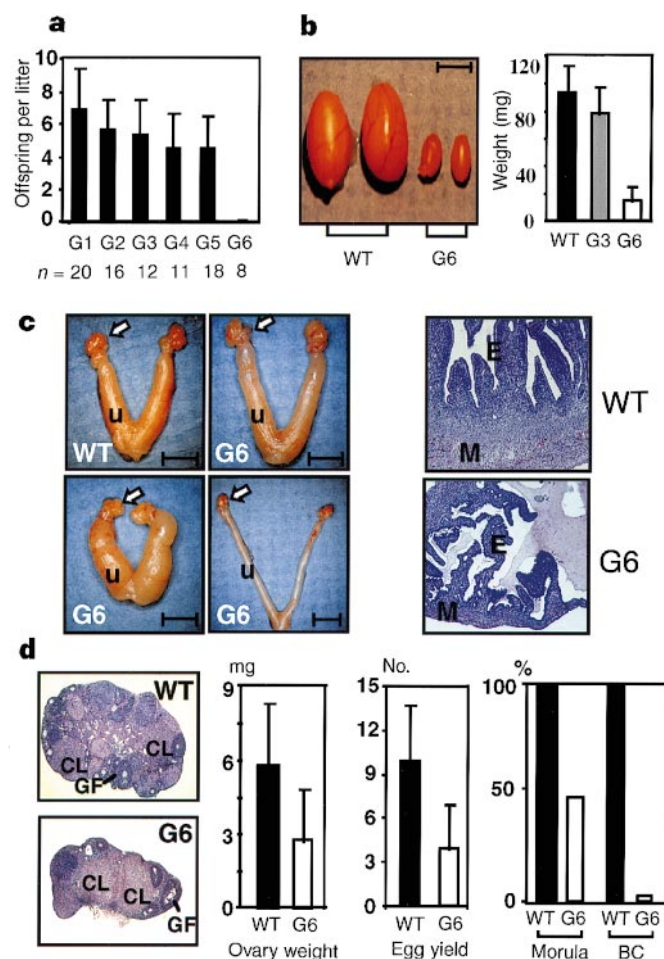


Figure 1 Effects of lack of telomerase on the mouse reproductive system. **a**, Impaired reproductive function in late-generation mTR -deficient mice. Males aged 6–12 weeks and females aged 4–12 weeks of the same generation of telomerase-deficient mice were mated over a period of 2 months or longer; n indicates the number of mating pairs examined. The P values of each generation were calculated compared with G_1 (G_2 , $P = 0.063$; G_3 , $P = 0.070$; G_4 , $P = 0.021$; and G_5 , $P = 0.004$). **b**, Testicular atrophy and testes weights. Scale bar, 5 mm. **c**, Uterine horns. Grossly dissected uterine horns (u) and ovaries (arrows) (scale bar, 5 mm) and haematoxylin and eosin (H&E) staining (magnification $\times 50$). **d**, Ovarian structure and function. Oestrus-matched ovaries are shown at $\times 50$ magnification (left). E, endometrium; M, myometrium; GF, Graafian follicle; CL, corpora lutea. The graphs show a weight comparison of ovaries, average egg yields following natural mating with a wild-type stud male, and the percentage of *in vitro* development of pre-implantation embryos. BC, blastocyst.

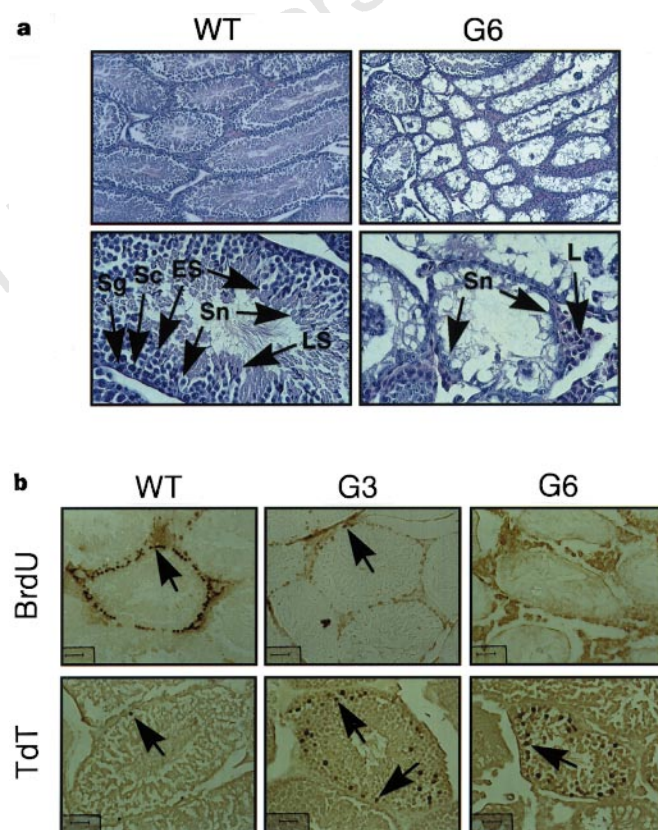


Figure 2 Proliferation and apoptosis in the male germ-cell compartment. **a**, Histology of WT and G_6 testis. Top: sections at low magnification ($\times 50$) through WT testes and representative G_6 testes show the diminished cellularity in the G_6 testes although there are some tubules undergoing nearly normal spermatogenesis (left edge of G_6 section). At higher magnification ($\times 200$ (bottom)), all stages of spermatogenesis are well represented in WT seminiferous tubules, whereas most G_6 tubules lack spermatogenesis yet retain Sertoli cells. In the G_6 testes, the apparent local accumulation of Leydig cells in the interstitium is secondary to contraction of the seminiferous tubules and an overall decrease in testis volume. Sn, Sertoli cell; L, Leydig cell; Sg, spermatogonia; Sc, primary spermatocyte; ES, early spermatid; LS, late spermatid. **b**, Comparative BrdU incorporation and TUNEL assays. Relative to WT samples, there was an approximate reduction in BrdU-positive nuclei (arrows) of three- to fivefold for G_3 mice and greater than 20- to 100-fold for G_6 mice. In the TUNEL (TdT) assay, G_3 seminiferous tubules and G_6 tubules with adequate cellularity for comparative analysis possessed many TUNEL-stained germ-cell nuclei (arrows), whereas only an occasional apoptotic cell was detected in the WT samples. Magnification $\times 100$.

divisions from zygote to mature sperm has been estimated to be >60, whereas oocytes undergo arrest and await maturation after only 25 cell divisions²⁶, we suspected that telomerase deficiency would be seen initially and most adversely in the male germ-cell compartment. On gross inspection, the size and weight of G₆ testes were found to be reduced by ~80% compared with age-matched wild-type or G₃ testes, whereas other organs (such as brain) or accessory reproductive organs (such as seminal vesicles) in G₆ animals were proportional to the overall body size (Fig. 1b). At the histological level, testes of wild-type through to G₄ mice possessed well-developed seminiferous tubules with a full complement of support cells (Sertoli cells) and germ cells representing all stages of spermatogenesis, similar to what is seen in wild-type age-matched controls (Fig. 2a). By the fifth generation, germ cells became depleted (data not shown) and, by the sixth generation, most seminiferous tubules

showed a striking absence of spermatogenesis yet maintained an apparently adequate representation of the less proliferative Sertoli and Leydig cells (Fig. 2a). Thus spermatogenic cells, not support/accessory cells, are particularly affected by telomerase deficiency in the G₆ *mTR*^{-/-} mice. As sexual behaviour and seminal-vesicle development and structure are highly dependent on adequate levels of androgens²⁷, the observations that male sexual performance (plugging efficiency) and seminal-vesicle size and structure were normal strongly indicate that germ-cell depletion is not a simple consequence of low serum testosterone levels (data not shown).

Progressive hypospermia could result from a checkpoint-arrest response and/or diminished viability (apoptosis) as late-generation germ cells lose telomere sequences and experience genomic instability. To investigate these possibilities, we monitored the rates of proliferation and apoptosis in wild-type, G₃, G₅ and G₆ testes. *In situ* assays of bromodeoxyuridine (BrdU) incorporation revealed abundant S-phase activity in many seminiferous tubules of the wild-type testes (Fig. 2b). In the histologically normal G₃ testes, a greater than fivefold decrease in the number of BrdU-positive nuclei was noted (Fig. 2b). This proliferative decline was more pronounced in the G₅ and G₆ testes, even in tubules with moderate cellularity (Fig. 2b; G₅ data not shown). Moreover, a marked increase in apoptosis (measured with TdT-mediated dUTP nick end labelling (TUNEL)) was detected in the G₃ and G₆ testes compared with wild-type controls (Fig. 2b; compare G₃ and G₆ with wild-type). Thus, at least two processes are responsible for progressive germ-cell depletion: decreased proliferation and increased apoptosis.

Female reproductive system. On gross examination, oestrus-staged G₆ uterine horns exhibited minimal to significant reductions in length and diameter compared with age- and stage-matched wild-type controls (Fig. 1c). G₆ ovaries also varied considerably in size and, on average, weighed markedly less than wild-type controls (Fig. 1d; wild-type 5.4 ± 2.5 mg, G₆ 2.6 ± 2.0 mg; *P* = 0.0212). Detailed histological analysis of G₆ uterine horns showed normal endometrial thickness, integrity and cellularity; however, myometrial thickness was reduced and atrophy of the smooth muscle cells was increased in the smaller and more contracted G₆ uterine horns (Fig. 1c). Although histological examination of the ovaries confirmed the reduced size of the G₆ ovary, both wild-type and G₆ ovaries showed a full spectrum of follicular development including Graafian follicles and corpora lutea (Fig. 1d).

To assess physiological aspects of reproduction, the plugging frequency and egg yields were measured after natural matings of G₆ or wild-type females with wild-type males. Under identical temporal and environmental conditions, 11 of 11 wild-type and 8 of 10 G₆ females were plugged and produced an average of 9.8 (± 3.8) and 3.9 (± 2.9) fertilized eggs per female, respectively (Fig. 1d, *P* = 0.0175). We then examined the capacity of these embryos to progress through early embryogenesis after culturing *in vitro*. Although all (15 of 15) wild-type/wild-type F₁ embryos developed to the blastocyst stage (Fig. 1d, *P* = 0.0061), only 46.7% (7 of 15) of G₇/wild-type F₁ embryos developed to the morula stage and none progressed to the blastocyst stage. To compare reproductive-tract function, wild-type fertilized eggs were implanted into the oviducts of G₆ or wild-type pseudopregnant females. Only 2 of 7 G₆ females were able to support the full-term development of these embryos compared with 8 of 10 wild-type females. Thus, although the comparable plugging efficiencies indicate normal hormonal regulation and end-organ responsiveness, infertility probably results from several independent factors. These include a decrease in the number of oocytes upon ovulation, poor early developmental progression of G₇ wild-type F₁ embryos, and possible compromise in uterine structure/function.

Haematopoietic system

Most haematopoietic cells have a lifespan considerably shorter than that of other somatic cells, and these cells are continually replen-

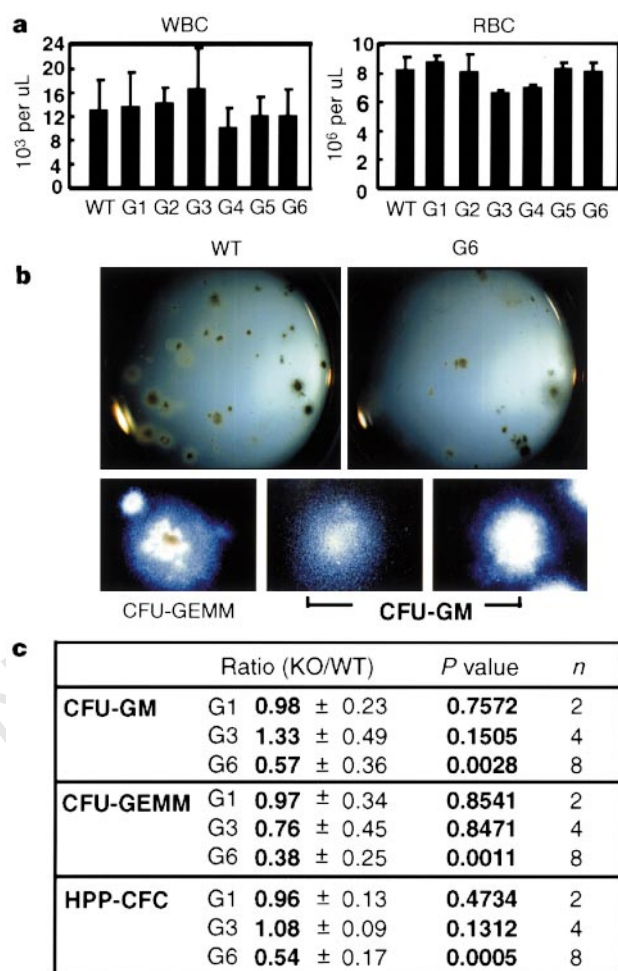


Figure 3 Effects of the lack of telomerase on haematopoietic cells. **a**, Peripheral blood counts for red blood cells (RBC) and white blood cells (WBC). Peripheral blood was subjected to the Coulter STK/S counter analysis for the complete blood count. **b**, Haematopoietic colony assays. Formation of bone-marrow-derived colonies was assessed *in vitro* using cells obtained from WT and from G₆ femurs. Representative colonies of CFU-GEMM and CFU-GM are shown (magnification ×10). HPP-CFC colonies are scored on the basis of size (see Methods). **c**, Ratios of numbers of knockout (KO) to WT colonies. *P* values were calculated from the comparison between numbers of WT and homozygous-null mice of each generation; *n* is the number of mice that were used for each experiment.

ished by a self-renewing populations of stem cells²⁸. A histological survey of adult bone marrow, spleen and thymus revealed normal organ size, architecture and cellularity through to the sixth generation (data not shown). A complete peripheral blood count and profile of sex- and age-matched mice did not show any differences in all genotypes through successive generations of telomerase deficiency (Fig. 3a). Moreover, no differences were observed in the percentages of lymphocytes, neutrophils, basophils, eosinophils and monocytes. Fluorescence-activated cell sorting (FACS) analysis of splenocytes showed identical cell-type profiles as measured by antibodies against T cells (antibodies against CD4, D8, and CD3), B cells (anti-B220), lymphoid cells (anti-CD45), macrophages (anti-F4/80), and erythrocytes (anti-TER-119) (data not shown). Similarly, FACS analyses of bone-marrow cells showed identical profiles using antibodies against D45, F4/80, TER-119 and CD34 (stem cell) (data not shown). To test the immune response of telomerase-deficient mice, six wild-type and six G_5 naive mice were infected intravenously with a sublethal inoculum of *Listeria monocytogenes*. The two groups showed similar survival and recovery rates. Upon rechallenge with a dose that would be lethal to naive mice, all mice survived, thus demonstrating that gross function of the immune response and memory is not affected. Mature haematopoietic organ structure and function seem to be well compensated in mTR -deficient mice.

As relatively few haematopoietic stem cells (HSCs) sustain long-term haematopoiesis, reductions in HSC numbers or growth/survival potential might not be reflected in peripheral blood count measurements. Thus we used *in vitro* haematopoietic

colony-forming unit (CFU) assays to determine whether the early progenitor-cell compartment was compromised. Bone-marrow cell suspensions derived from G_1 and G_5 $mTR^{-/-}$ mice were comparable to WT suspensions in their ability to generate CFU-granulocyte, monocyte (CFU-GM), CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and high-proliferative-potential colony-forming cell (HPP-CFC) colonies (Fig. 3c). In contrast, multiple CFU assays comparing wild-type, G_1 , G_3 and G_6 progenitor-cell profiles showed a statistically significant decrease in the total number of CFU-GM ($P = 0.0028$), CFU-GEMM ($P = 0.0011$) and HPP-CFC ($P = 0.0005$) colonies in the G_6 samples (Fig. 3b, c). These results indicate that the long-term renewal of haematopoietic stem cells is compromised upon telomere loss. Nevertheless, the stochastic growth behaviour of these cells may allow the generation of a reserve pool that is still capable of populating the peripheral haematopoietic compartment.

Lymphocyte mitogenic response

Telomerase activity is low or undetectable in normal human B and T cells and increases dramatically after *in vitro* mitogenic stimulation^{29,30}. This transient increase in telomerase activity indicates that somatic activation of telomerase may delay replicative senescence and facilitate mitogen-induced clonal expansion³¹. To determine the effects of telomerase deficiency on the ability of mature lymphocytes to proliferate, we exposed splenocytes, isolated from wild-type through to G_6 $mTR^{-/-}$ mice, mitogens that mainly activate T cells (phorbol myristate acetate (PMA) plus ionomycin (Ion), or anti-CD3/CD28 antibodies, or a more general T-cell-mitogenic activator (Concanavalin A (Con A)). For the three mitogenic stimuli, splenocytes derived from wild-type or G_1 through to G_4 $mTR^{-/-}$ mice showed almost identical degrees of uptake of 3H -labelled thymidine, although a decrease first became apparent in an occasional G_4 mouse (data not shown). In contrast, G_5 (four mice) and G_6 (three mice) stimulated $mTR^{-/-}$ splenocytes exhibited a statistically significant decrease in incorporation of 3H -labelled thymidine compared with wild-type (seven mice) controls (Fig. 4a). Incorporation of 3H -labelled thymidine in G_6 $mTR^{-/-}$ splenocytes was ~32% with Con A, ~38% with PMA/Ion, and ~39% with anti-CD3/CD28 antibodies relative to wild-type controls (Fig. 4a).

To determine the basis for the decreased incorporation of 3H -labelled thymidine, we assayed the cell-cycle profile and the percentage of apoptosis after PMA/Ion stimulation of wild-type and G_6 splenocytes. Although wild-type and G_6 cell-cycle profiles were nearly identical after stimulation, a marked increase in the percentage of late apoptotic cells (annexin-positive and propidium iodide (PI)-positive) was observed in the G_6 splenocytes; this increase was inversely proportional to the decrease in incorporation of 3H -labelled thymidine (Fig. 4b). The normal mitogenic response of earlier generation $mTR^{-/-}$ splenocytes and the pronounced and progressive effect on the later generations indicates that, although telomerase does not play a direct role in splenic lymphocyte activation and expansion, telomere shortening and chromosomal aberrations in late generations may lead to induction of apoptosis, possibly through either DNA-damage-response pathways or significant chromosome loss or rearrangement after mitogenic stimulation.

Chromosome analysis

To examine whether the impaired mitogenic (pro-apoptotic) response of cultured lymphocytes could be related to significant telomere shortening and genomic instability, PMA/Ion- or Con A-activated splenocytes from two wild-type and six G_6 $mTR^{-/-}$ mice were treated with colcemid and karyotyped by G-banding. The percentage of activated cells with an abnormal chromosome count (aneuploidy) was 16.7% (7 of 42) for $mTR^{+/+}$ and 37.7% (53 of 144) for G_6 samples (Table 1a). In contrast, control metaphase spreads of

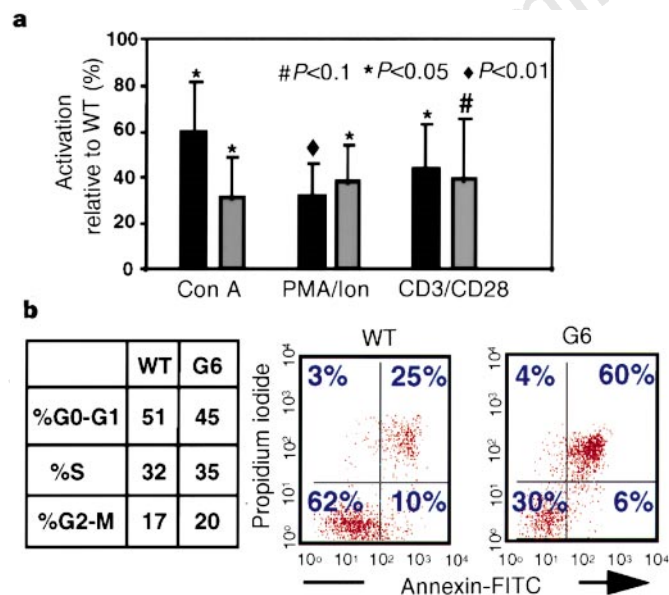


Figure 4 Response of lymphocytes to lack of telomerase. **a**, Splenocyte mitogenic response. Splenocytes from WT, G_5 and G_6 homozygous-null animals were mitogenically activated and their uptake of 3H -labelled thymidine was measured. The y-axis represents the percentage of fold-activation of mTR -deficient splenocytes by indicated mitogens (x-axis) compared with activation of WT splenocytes. Mitogenic activation of WT splenocytes was set at 100%. Error bars indicate standard deviations from quadruplicated samples of four G_5 mice and three G_6 mice. Black bars show the data from G_5 mice and grey bars indicate data from G_6 mice. **b**, Cell-cycle profile and annexin/PI-stained FACS profiles. Cell-cycle profiles are shown at the left. Percentages of the early and late stages of apoptotic cells are shown at the right. There were approximately 30% more G_6 apoptotic splenocytes (annexin-positive) than WT apoptotic splenocytes 48 h after activation by PMA/Ion.

Table 1 Cytogenic analysis

a Number of chromosomes per metaphase	Number of metaphases	
	WT	G ₆
>40	1	12
40 (normal)	35	91
39	1	26
38	1	7
37	1	4
36	1	0
35	1	1
34	0	1
33	0	3
32	1	0
31	0	2
Total number of metaphases examined	42	155
Aneuploidy (%)	16.7	37.7

b Sample number	Chromosome involved in fusions	
	WT	G ₆
1	t(1;1)	t(3;3)
2	t(X;#)	t(3;3)
3		t(3;3)
4		t(3;13)
5		t(3;14)
6		t(13;13)
7		t(14;14)

a, Metaphase spreads derived from activated WT and G₆ splenocytes were examined for abnormal chromosome number (aneuploidy). Percentages of aneuploidy were calculated by the number of metaphases carrying abnormal chromosome number (not 40) divided by the total number of metaphases examined. **b**, Chromosome numbers that participated in the fusions were identified by G-banding and listed. All fusions seemed to be the Robertsonian type and the short (p) arms were involved in all cases. #, not identifiable.

mTR^{+/+} mouse embryonic fibroblast (MEF) cells showed no (0 of 17) aneuploidy (data not shown); the high baseline aneuploidy in activated *mTR*^{+/+} splenocytes is not understood. When splenocyte metaphase spreads were scored for abnormal chromosome structures, 3.6% (2 of 55) of wild-type and 21.1% (40 of 190) of G₆ samples had chromosomes with abnormal G-banding patterns. We found end-to-end fusions that seem to be of the Robertsonian type^{32,33}.

To identify the specific chromosomes involved in the fusions, we conducted chromosomal G-banding on randomly selected G₆ metaphases and on the sole wild-type metaphases exhibiting fusion. In seven informative cases for the activated G₆ splenocytes, chromosomes 3, 13 and 14 were involved in the fusion events. This differed from the fusions in the two wild-type samples, where chromosomes 1 and X were involved (Table 1b). Although the frequent involvement of chromosome 3 in the G₆ samples (5 out of 7 fusions; Table 1b) is intriguing and could relate to the unusually short telomeres of its short (p) arm³⁴, the biological significance of this is unknown. Studies of human tumours and ageing cells did not show correlations between telomere length and frequency of involvement in fusion events for a given chromosome³⁵.

Finally, analysis of telomere length in several tissues by Southern blotting did not show any significant differences between the different generations or among tissues of the same generation (data not shown). In contrast, fluorescence *in situ* hybridization (FISH) using a probe for telomere repeat DNA generated fewer than four end signals in only a few chromosomes (~7.5%) in the wild-type metaphase spreads, whereas far more chromosomes in G₆ *mTR*^{-/-} cells exhibited fewer than four signals (~58.75%) (data not shown). As all Robertsonian fusions in the *mTR*^{-/-} sample failed to generate a signal at their junctions, these fusion events seem to be caused by the loss of telomere function at these chromosomes.

Discussion

In dividing cells, telomerase is essential in telomere maintenance and, in its absence, significant erosion of telomeres is thought to

signal replicative senescence and eventually lead to a crisis period highlighted by genetic instability and loss of cell viability. The presence of telomerase activity in germ cells and highly proliferative stem-cell compartments, and its upregulation in activated T lymphocytes, indicate that telomerase may be required for the proliferation and long-term viability of such cells. Here we demonstrate that telomerase deficiency leads to a depletion of male germ cells, diminished haematopoietic colony formation, and impaired mitogen-induced proliferation of primary splenocytes.

In the testes, the significant decline in BrdU incorporation and increase in TUNEL-positive nuclei suggests that telomere shortening leads to both checkpoint and apoptotic responses *in vivo*. In T cells, mitogenic stimulation of cells with short telomeres led to cytogenetic abnormalities and increased apoptosis. These results indicate that telomerase is essential for the long-term maintenance of diverse cell types with high proliferation profiles, and suggest that alterations in telomere structure dramatically affect the number, growth potential and/or survival of renewing cell populations. Marked aneuploidy and Robertsonian fusions were observed with high frequency in activated splenocytes derived from G₆ *mTR*^{-/-} mice, a finding similar to that reported for late passage *mTR*^{-/-} MEF culture²⁰. These chromosomal anomalies were associated with a striking absence in telomere-repeat signal on a pronounced number of chromosome ends, further substantiating the concept that telomeres are vital in maintaining chromosomal integrity and genomic stability in normal cells *in vivo*.

The delay of several generations in emergence of a phenotype parallels the lag period observed in telomerase-deficient yeast cells, in which viability was compromised only after many cell divisions. The long latency in the appearance of defects in the mouse may simply relate to the long telomere lengths of the laboratory mouse strain *Mus musculus*³⁷. Once significant telomere loss is reached, some cell types seem to be more adversely affected than others. The basis for the more prominent phenotype in the haematopoietic and germ cells could relate to a higher level of cell turnover and resultant greater telomere loss; however, other highly proliferative organs such as the intestine appear to be unaffected. Some cell types might be intrinsically less tolerant of genetic instability, perhaps reflecting a strategy to ensure faithful transmission of genetic information in the case of sperm cells or to guard against haematopoietic malignancies over a lifetime of intense proliferation. A detailed comparison of telomere lengths and karyotypes among these various cell types will be necessary to better resolve this issue.

Telomerase deficiency ultimately produced a reduction in proliferation and an increase in apoptosis in the mouse male germ cells. Such a response as the reduction of telomeres to 'critical length' in cultured human cells correlates with cessation of proliferation and maintenance of a viable senescent state. Human cells will remain in this state unless this checkpoint is commandeered by viral oncoproteins or genetic mutation. The difference between mice and humans may be related to the fact that the rodent cells find it easier to escape from the slow-growth 'senescent' phase than do human cells, the growth of which is tightly regulated. Thus the mouse cells continue more readily towards crisis.

These studies demonstrate that, although telomerase activity *per se* does not play a direct biological role in mammalian development, telomerase is vital in cell survival and organ homeostasis through maintenance of adequate telomere structure during cell division. The mouse model described here provides a system with which to understand the complexities of telomere dynamics and how they relate to cell physiology. These studies provide new insights into basic telomere biology and help us anticipate the systemic effects of telomerase inhibitors in cancer therapy. The maintenance of normal peripheral blood cell counts, immune competence and gastrointestinal function in the telomerase-deficient mouse indicates that diverse stem-cell compartments may sustain organ function after systemic administration of telomerase inhibitors. The high

proliferative index of most cancer cells compared with the more sporadic cycling of normal stem-cell populations suggests that telomerase inhibition will be well tolerated in clinical settings. □

Methods

Mice. The genetic background of the mice (WW6/C57BL/6J) is identical to that of our previous work²⁰. Age- and sex-matched animals were used in all experiments, with ages ranging from 6 to 14 weeks old. For analysis of female reproductive system, females 6–14 weeks old of the indicated genotypes were mated with CBA/B6 F₁ stud males. Fertilized eggs were cultured in KSOM media (Specialty).

Haematopoietic colony assay. Murine colony assay was performed according to the manufacturer's recommendation (StemCell Technologies). The colony counts for CFU-GM were performed on days 7–10 and for CFU-GEMM and HPP-CFC on days 12–14. HPP-CFC was defined as those colonies measuring 0.5 mm on day 12–14 (ref. 38).

Incorporation of ³H-labelled thymidine and FACS analyses. Splenocytes were resuspended in RPMI1640/10% FBS/0.55 μM β-mercaptoethanol. The mitogens used (Sigma or Pharmingen) were as follows: PMA (20 nM), ionomycin (1 μM), Con A (4 μg ml⁻¹), hamster anti-mouse CD3e (10 ng ml⁻¹) and hamster anti-mouse CD28 (10 ng ml⁻¹). After a pulse of ³H-labelled thymidine, the cells were collected at 48 h following the addition of mitogen. The incorporated c.p.m. was determined by a liquid scintillation counter. We processed samples in quadruplicate and calculated averages, standard deviations and *P* values. To analyse haematopoietic subpopulations by FACS, cells from each organ were incubated with antibodies (Pharmingen or Caltag) and analysed by FACSscan (Becton Dickinson Immunocytometry Systems; LYSIS II). The antibodies were CD45-PE, IgM-PE, CD4-PE, CD3-fluorescein isothiocyanate (FITC), B220-FITC, CD8-ITC and MAC-1-FITC.

Cell-cycle analysis and apoptosis assay. Splenocytes were activated by PMA/Ion for 48 h. Cell-cycle and apoptosis assays were then used. For cell-cycle analysis we modified the method in ref. 39. For apoptosis assays, we used annexin-V-Fluos (Boehringer) to detect the apoptotic cell population according to the manufacturer's recommendation. For tissue sections, *in situ* apoptosis and BrdU incorporation assays were performed as described⁴⁰.

Cytogenetic and FISH analyses. Metaphase spreads of splenocytes were prepared and processed for G-banding by standard methods⁴¹ following activation by Con A or PMA/Ion and colcemid treatment. For FISH analysis, hybridization of FITC-conjugated (TTAGGG)_n (Oncor) was performed according to the manufacturer's recommendations.

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- Greider, C. W. Telomere length regulation. *Annu. Rev. Biochem.* **65**, 337–365 (1996).
- Müller, H. J. The remaking of chromosomes. *Collect. Net* **8**, 182–195 (1938).
- McClintock, B. The stability of broken ends of chromosomes in *Zea mays*. *Genetics* **26**, 234–282 (1941).
- Greider, C. W. Chromosome first aid. *Cell* **67**, 645–647 (1991).
- Greider, C. W. & Blackburn, E. H. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* **43**, 405–413 (1985).
- Greider, C. W. & Blackburn, E. H. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* **337**, 331–337 (1989).
- Feng, J. *et al.* The RNA component of human telomerase. *Science* **269**, 1236–1241 (1995).
- Lingner, J. *et al.* Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**, 561–567 (1997).
- Harrington, L. *et al.* Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev.* **11**, 3109–3115 (1997).
- Kilian, A. *et al.* Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns on different cell types. *Hum. Mol. Genet.* **6**, 2011–2019 (1997).

- Nakayama, J. *et al.* Telomerase activation by hTERT in human normal fibroblasts and hepatocellular carcinomas. *Nature Genet.* **18**, 65–68 (1998).
- Meyerson, M. *et al.* HEST2, the putative human telomerase catalytic subunit gene, is upregulated in tumor cells and during immortalization. *Cell* **90**, 785–795 (1997).
- Nakamura, T. M. *et al.* Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**, 955–959 (1997).
- Harley, C. B., Futcher, A. B. & Greider, C. W. Telomeres shorten during aging of human fibroblasts. *Nature* **345**, 458–460 (1990).
- Hastie, N. D. *et al.* Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**, 866–868 (1990).
- Bodnar, A. G. *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352 (1998).
- Counter, C. M. *et al.* Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**, 1921–1929 (1992).
- Prowse, K. R. & Greider, C. W. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl Acad. Sci. USA* **92**, 4818–4822 (1995).
- Kim, N. W. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015 (1994).
- Blasco, M. A. *et al.* Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25–34 (1997).
- Singer, M. S. & Gottschling, D. E. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**, 403–409 (1994).
- McEachern, M. J. & Blackburn, E. H. Runaway telomere elongation caused by telomerase RNA gene mutations. *Nature* **376**, 403–409 (1995).
- Haber, J. E. & Thorburn, P. C. Healing of broken linear dicentric chromosomes in yeast. *Genetics* **106**, 207–226 (1984).
- Sandell, L. L. & Zakian, V. A. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* **75**, 729–739 (1993).
- Wright, S. Systems of mating. *Genetics* **6**, 111–178 (1921).
- Drost, J. B. & Lee, W. R. Biological basis of germline mutation: comparisons of spontaneous germline mutation rates among *Drosophila*, mouse, and human. *Environ. Mol. Mutagen.* **25** (suppl. 26), 48–64 (1995).
- Shima, H., Motomu, T., Young, P. & Cunha, G. R. Postnatal growth of mouse seminal vesicle is dependent on 5α-dihydrotestosterone. *Endocrinology* **127**, 3222–3233 (1990).
- Erslev, A. J. & Lichtman, M. A. in *Hematology* 4th edn 37–47 (McGraw-Hill, 1993).
- Buchkovich, K. J. & Greider, C. W. Telomerase regulation during entry into the cell cycle in normal human T cells. *Mol. Biol. Cell* **7**, 1443–1454 (1996).
- Weng, N.-P., Levine, B. L., June, C. H. & Hodes, R. J. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J. Exp. Med.* **183**, 2471–2479 (1996).
- Bodnar, A. G., Kim, N. W., Effros, R. B. & Chiu, C. P. Mechanism of telomerase induction during T cell activation. *Exp. Cell Res.* **228**, 58–64 (1996).
- Garagna, S. *et al.* Robertsonian metacentrics of the house mouse lose telomeric sequences but retain some minor satellite DNA in the pericentromeric area. *Chromosoma* **103**, 685–692 (1995).
- Nanda, I., Schneider-Rasp, S., Winking, H. & Schmid, M. Loss of telomeric sites in the chromosomes of *Mus musculus domesticus* (Rodentia: Muridae) during Robertsonian rearrangement. *Chromosome Res.* **3**, 399–409 (1995).
- Zijlmans, J. M. *et al.* Telomeres in the mouse have large inter-chromosomal variations in the number of T₂AG₃ repeats. *Proc. Natl Acad. Sci. USA* **94**, 7423–7428 (1997).
- Saltman, D., Morgan, R., Cleary, M. L. & de Lange, T. Telomeric structure in cells with chromosome end associations. *Chromosoma* **102**, 121 (1993).
- McEachern, M. J. & Blackburn, E. H. Cap-prevented recombination between terminal telomeric repeat arrays (telomere CAPR) maintains telomeres in *Kluyveromyces fragilis* lacking telomerase. *Genes Dev.* **10**, 1822–1834 (1996).
- Kipling, D. & Cooke, H. J. Hypervariable ultra-long telomeres in mice. *Nature* **347**, 400–402 (1990).
- Watt, S. M. & Visser, J. W. M. Recent advances in the growth and isolation of primitive human haemopoietic progenitor cells. *Cell Prolif.* **25**, 263–297 (1992).
- Vindelov, L. L., Christensen, I. J. & Nissen, N. L. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3**, 323–327 (1983).
- Morgenbesser, S. D. *et al.* P53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371**, 72–74 (1994).
- Cannizzaro, L. A. & Shi, G. Fluorescent *in situ* hybridization (FISH) for DNA probes in the interphase and metaphase stages of the cell cycle. *Methods Mol. Biol.* **75**, 313–322 (1997).

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Large convection cells as the source of Betelgeuse's extended atmosphere

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Supergiant stars such as Betelgeuse have very extended atmospheres, the properties of which are poorly understood. Alfvén waves^{1–4}, acoustic waves^{1,2,5–7} and radial pulsations⁸ have all been suggested as likely mechanisms for elevating these atmospheres and driving the massive outflows of gas seen in these stars: such mechanisms would heat the atmosphere from below, and there are indeed observations showing that Betelgeuse's extended atmosphere is hotter than the underlying photosphere^{9,10}. Here we report radio observations of Betelgeuse that reveal the temperature structure of the extended atmosphere from two to seven times the photospheric radius. Close to the star, we find that the atmosphere has an irregular structure, and a temperature ($3,450 \pm 850$ K) consistent with the photospheric temperature but much lower than that of gas in the same region probed by optical and ultraviolet observations¹⁰. This cooler gas decreases steadily in temperature with radius, reaching $1,370 \pm 330$ K by seven stellar radii. The cool gas coexists with the hot chromospheric gas, but must be much more abundant as it dominates the radio emission. Our results suggest that a few inhomogeneously distributed large convective cells (which are widely believed^{11–16} to be present in such stars) are responsible for lifting the cooler photospheric gas into the atmosphere; radiation pressure on dust grains that condense from this gas may then drive Betelgeuse's outflow.

We observed Betelgeuse for 11 hours on 21 December 1996 with the Very Large Array (VLA) in its highest-resolution (A) configuration. At a wavelength of 7 mm, the angular resolution achieved is sufficient to have resolved Betelgeuse's optical disk, which subtends the largest angular diameter of any star visible in the night sky from the Northern Hemisphere. Because Betelgeuse's radio emission is purely thermal and optically thick (as demonstrated below), such spatially resolved radio observations also act as a pure thermometer of the stellar atmosphere. This is in contrast to observations in the ultraviolet continuum or optical and ultraviolet spectral lines, where the gas temperature is derived by comparison with theoretical model atmospheres. The VLA is at present equipped with 7-mm receivers on 13 of its 27 antennas. We used the remaining 14 antennas to observe at the longer wavelengths of 1.3, 2, 3.6 and 6 cm, where we found Betelgeuse to be partially resolved. Figure 1 shows the final image obtained at 7 mm. Betelgeuse's atmosphere becomes opaque at very different heights when observed at different wavelengths, and at 7 mm the average diameter is approximately twice that measured in the optical. The radio surface is clearly not spherically symmetric, nor does it appear to be axially symmetric.

To quantify the total flux and area of the 7-mm surface and hence its brightness temperature, we fitted circular and elliptical disks to the measured visibilities. The best fit was provided by a uniformly bright ellipse with dimensions of 95 ± 2 mas $\times$ 80 ± 2 mas at a position angle of $67^\circ \pm 7^\circ$ (measured east from north), and a total flux density of 28.0 ± 5.6 mJy; the uncertainty in the flux measurements is dominated by the uncertainty in the absolute flux calibration of the VLA at 7 mm, estimated to be $\pm 20\%$. Although this

model ellipse does not provide a perfect fit to the observed disk structure, the residuals are small and approximately cancel in flux indicating that the total flux and area of the fitted ellipse nevertheless closely match that of the observed stellar radio disk. The implied brightness temperature is $3,450 \pm 850$ K (the uncertainty here, dominated by the uncertainty in the absolute flux calibration, represents extreme limits; elsewhere the quoted uncertainty refers to $\pm 1\sigma$), consistent with the stellar photospheric temperature of $\sim 3,600$ K (ref. 17). At the longer radio wavelengths—where Betelgeuse's atmosphere appears increasingly large while our angular resolution becomes progressively poorer—we derived just the average diameter and total flux density of the stellar radio disk by fitting uniformly bright circular disks to the measured visibilities at each wavelength. The increase in size with radio wavelength implies that the observed radio surfaces are optically thick, and hence have brightness temperatures reflecting the local gas temperature. In Fig. 2 we plot the measured brightness temperature of Betelgeuse's atmosphere as a function of stellar radius. The temperature of the atmosphere can be seen to decrease steadily with increasing radius from $3,450 \pm 850$ K at $\sim 2R_*$ to $1,370 \pm 330$ K at $\sim 7R_*$ (here R_* is the radius of the photosphere).

Our results are at odds with current theoretical and empirical models for the structure and temperature of Betelgeuse's atmosphere. Theoretical models which invoke the dissipation of Alfvén waves^{1–4}, shocks produced by acoustic waves^{1,2,5–7}, or shocks produced by

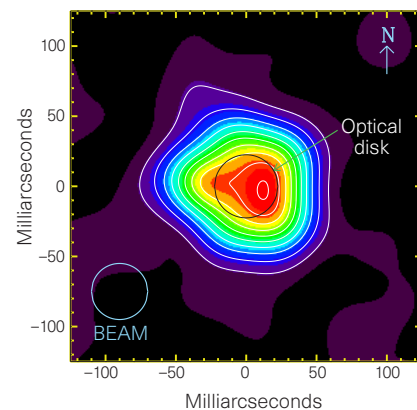


Figure 1 An image of Betelgeuse's atmosphere observed at a wavelength of 7 mm with the VLA. The angular resolution of this observation is 40 mas (blue circle at bottom left corner). White contour lines are plotted at 10%, 20%, ..., 90%, and 99% of the peak flux of the false-colour radio image. Betelgeuse's 7-mm surface has an average diameter approximately twice as large as its optical surface of diameter 45 mas (black circle with its centre placed coincident with the intensity-weighted centre of the radio disk), and is clearly not spherically symmetric. To make this image, we removed fast tropospheric phase fluctuations using a newly implemented "fast-switching" observing mode²⁴ at the VLA which allowed us to observe a nearby calibrator frequently without losing too much observing time. We therefore switched between Betelgeuse and an unresolved extragalactic object, 05528 + 03135 (used as our calibrator), located 4.2° away, with a cycle time of only ~ 2.5 min. To check the effectiveness of the amplitude and phase corrections, we also switched between 05528 + 03135 and another unresolved extragalactic object, 05326 + 07327 (used as our control source), located 5.3° away, at two intervals during the observation with the same cycle time; this yielded an unresolved image for the control source. We set the flux density scale using the primary flux calibrator 3C286. The final image includes a single iteration in phase self-calibration to correct for small residual phase errors: this self-calibrated image has a $\sim 60\%$ lower noise fluctuation level than the original image (consistent with the overall system noise), but is morphologically identical. Also, the final image was constructed using natural weighting of the visibilities but restored with a synthesized beam of size corresponding to that obtained using uniform weighting. The uniform weighted image is identical except for a slightly (35%) higher noise level, demonstrating the robustness of our conservative implementation of the super-resolution technique.

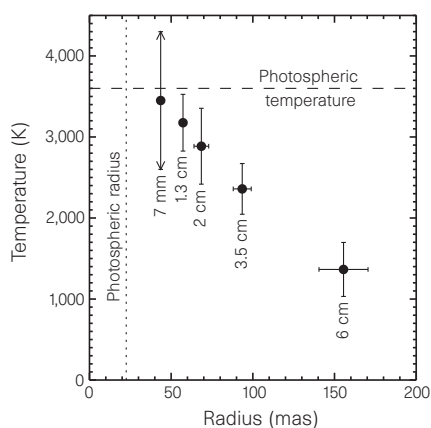


Figure 2 The temperature profile of Betelgeuse's atmosphere measured at the radio wavelengths indicated below each point. All the points have $\pm 1\sigma$ error bars, except for the 7-mm data point where the error bar with arrows indicate extreme limits (see text). The photospheric radius of 22.5 mas (ref. 17) is indicated by a dotted line, and the photospheric temperature of 3,600 K (ref. 17) is indicated by a dashed line. The stellar radius at 7 mm corresponds to the average radius measured in the spatially resolved image of Fig. 1. At the longer radio wavelengths, we derived the stellar radius by fitting a uniformly bright circular disk to the measured visibilities at each wavelength. The brightness temperature at each wavelength was calculated using the equation $T_b = 2.0 S_{\text{maj}} (\lambda_{\text{cm}})^2 / (\theta_{\text{maj}} \theta_{\text{min}})$ K, where S_{maj} is the total flux density, λ_{cm} is the observing wavelength, and θ_{maj} and θ_{min} are the major and minor axes of the stellar radio disk, respectively, in arcseconds. We note that the derived brightness temperature is independent of any assumptions such as the distance to the star.

radial pulsations⁸ to elevate the stellar atmosphere all predict a globally spherically symmetric structure, contrary to that in fact observed. Empirical models constructed to fit previous ultraviolet and radio data predict a spatially extended hot chromosphere^{18,19}, consistent with the idea that strong heating at low heights elevates the stellar atmosphere. The ultraviolet image of Betelgeuse taken with the Hubble Space Telescope (HST) clearly shows its chromosphere to have a diameter of 125 ± 6 mas in the continuum at 2,550 Å, which is inferred to be formed at a temperature of $\sim 5,000$ K (ref. 10). Our radio measurements, however, show that the hot chromospheric gas is not the only component at this height in Betelgeuse's atmosphere. The 2,550 Å height is above that which we probed at 7 mm, but is almost identical to that probed at 1.3 cm (diameter of 114 ± 4 mas). The temperature measured at 1.3 cm, however, is only $3,180 \pm 350$ K, formally below the photospheric temperature. From scans of the ultraviolet disk with the HST, the chromosphere observed in the Mg II h and k emission lines is inferred to extend to an even larger diameter of at least 270 mas (ref. 9). This surface is roughly comparable in diameter to that which we and others¹⁹ have probed at 6 cm (diameter of 310 ± 30 mas), but here the temperature measured in radio is just $1,370 \pm 330$ K. An earlier speckle image of Betelgeuse's chromosphere observed in the H α absorption line, which should be formed at a slightly higher temperature than the 2,550 Å continuum, showed a diameter of ~ 95 mas (ref. 20). This is comparable in diameter to our 7-mm measurement, but again the temperature measured in radio is much lower than is required for H α absorption. Clearly, there are significant differences over the same height range between the temperature of the atmosphere inferred from optical and ultraviolet observations and the temperature directly measured in our radio observations.

We therefore conclude that the relatively cool gas responsible for the observed stellar radio emission spans the same height range as the hotter gas responsible for the optical and ultraviolet chromospheric signatures. To determine the conditions under

which the cooler gas component could dominate the observed radio emission, we refer to the recent work by Reid and Menten²¹ who studied the radio opacity of late-type giant star atmospheres. They showed that the radio opacity of gas at a temperature significantly below 4,000 K is provided by the interactions of electrons, obtained predominantly from elements with low ionization potentials such as potassium and sodium, with neutral atomic and molecular hydrogen. At temperatures above 4,000 K, corresponding to that of the hot chromospheric gas, scattering of electrons by protons (that is, ionized hydrogen atoms) provides the dominant source of radio opacity. As the electron-neutral absorption per neutral particle is a factor of about 10^3 smaller than the electron-ion free-free absorption per ion, the preferential detection of the cooler gas component in our radio observations implies that it must be more than 3 orders of magnitude more abundant than the chromospheric gas. Under these circumstances, it seems unlikely that the diffuse chromospheric gas plays an important role in elevating Betelgeuse's atmosphere.

Instead, the irregular structure and predominantly low temperatures of Betelgeuse's atmosphere suggest the following alternative mechanisms for elevating its atmosphere and driving its mass outflow. Over 20 years ago, Schwarzschild¹¹ proposed that late-type giant and supergiant stars possess very large convection cells, a few of which may be present on the stellar surface at any one time. The existence of such large convection cells has since gained wide acceptance in order to explain the polarized optical light commonly seen from these stars (see ref. 12 and references therein), as well as photospheric bright spots seen on the few red supergiants imaged^{13–16}, most notably Betelgeuse. Optical images of Betelgeuse show that the morphology of its photosphere changes with time, but at any given time can be well fitted by the superposition of one or more bright spots (which are not always required) on a circular stellar disk^{13–16}. The HST ultraviolet image of Betelgeuse's atmosphere also appears to show a bright spot superposed on a circular disk¹⁰. Such large convection cells could elevate photospheric material into the stellar atmosphere^{11,16} and, if distributed inhomogeneously over the stellar surface, produce an asymmetric atmospheric structure.

The elevation of photospheric material into the stellar atmosphere naturally explains our measurements of photospheric-like temperatures at small stellar radii, while the smooth decrease in temperature with increasing radius may represent expansion and cooling of the elevated material. Radiation pressure on dust grains condensed from the dense cool gas could then drive Betelgeuse's massive outflow. This mechanism has long been postulated for driving the massive cool outflows of late-type giant and supergiant stars²², but faces severe difficulties if an extended inner region of the stellar atmosphere is entirely heated to chromospheric temperatures. Dust grains would then be required to form at relatively large stellar radii, where the gas density may in fact be too low for dust to condense effectively, or where radiation pressure may not be sufficiently intense to drive a massive outflow even if dust formation is possible. These problems are alleviated on Betelgeuse by the presence of dense cool gas in its inner atmosphere; indeed, dust has been detected to form episodically at radii as small as $\sim 4R_*$ (ref. 23). We note that the vigorous expulsion of gas into Betelgeuse's atmosphere would be expected to produce shock waves¹⁶, which could heat localized but spatially distributed (and therefore extended) regions of its atmosphere to chromospheric temperatures. $\square$

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- Hartmann, L. & MacGregor, K. B. Momentum and energy deposition in late-type stellar atmosphere and winds. *Astrophys. J.* **242**, 260–282 (1980).
- Hartmann, L. & MacGregor, K. B. Wave-driven winds from cool stars. I. Some effects of magnetic field geometry. *Astrophys. J.* **247**, 264–268 (1982).
- Holzer, T. E., Flå, T. & Leer, E. Alfvén waves in stellar winds. *Astrophys. J.* **275**, 808–835 (1983).
- Hartmann, L. & Avrett, E. H. On the extended chromosphere of α Orionis. *Astrophys. J.* **284**, 238–249 (1984).
- Ulmschneider, P. The chromospheric emission from acoustically heated stellar atmospheres. *Astron. Astrophys.* **222**, 171–178 (1989).

6. Cuntz, M. Chromospheric extents predicted by time-dependent acoustic wave models. *Astrophys. J.* **349**, 141–149 (1990).
7. Cuntz, M. On the generation of mass loss in cool giant stars due to propagating shock waves. *Astrophys. J.* **353**, 255–264 (1990).
8. Bowen, G. H. Dynamical modelling of long-period variable star atmospheres. *Astrophys. J.* **329**, 299–317 (1988).
9. Gilliland, R. & Dupress, A. K. *Stellar Surface Structure* (eds Strassmeier, K. G. & Linsky, J. L.) 165–172 (IAU Symp. No. 176, Kluwer, Dordrecht, 1996).
10. Gilliland, R. & Dupree, A. K. First image of the surface of a star with the Hubble Space Telescope. *Astrophys. J.* **436**, L29–L32 (1996).
11. Schwarzschild, M. On the scale of photospheric convection in red giants and supergiants. *Astrophys. J.* **195**, 137–144 (1991).
12. Doherty, L. R. On the polarization of Alpha Orionis. *Astrophys. J.* **307**, 261–268 (1986).
13. Buscher, D. F., Haniff, C. A., Baldwin, J. E. & Warner, P. J. Detection of a bright feature on the surface of Betelgeuse. *Mon. Not. R. Astron. Soc.* **245**, 7P–11P (1990).
14. Wilson, R. W., Baldwin, J. E., Buscher, D. F. & Warner, P. J. High-resolution imaging of Betelgeuse and Mira. *Mon. Not. R. Astron. Soc.* **257**, 369–376 (1992).
15. Klückers, V. A., Edmunds, M. G., Morris, R. H. & Woeder, N. Reality and the speckle imaging of stellar surfaces—II. The asymmetry of Alpha Orionis. *Mon. Not. R. Astron. Soc.* **284**, 711–716 (1997).
16. Tuthill, P. G., Haniff, C. A. & Baldwin, J. E. Hotspots on late-type supergiants. *Mon. Not. R. Astron. Soc.* **285**, 529–539 (1997).
17. Dyck, H. M., Benson, J. A., van Belle, G. T. & Ridgway, S. T. Radii and effective temperatures of K and M giants and supergiants. *Astron. J.* **111**, 1705–1712 (1996).
18. Skinner, C. J. & Whitmore, B. The circumstellar environment of α Orionis. *Mon. Not. R. Astron. Soc.* **224**, 335–348 (1987).
19. Skinner, C. J. *et al.* Circumstellar environments—V. The asymmetric chromosphere and dust shell of α Orionis. *Mon. Not. R. Astron. Soc.* **288**, 295–306 (1997).
20. Hebdin, J. C., Eckart, A. & Hege, E. K. The H α chromosphere of Alpha Orionis. *Astrophys. J.* **314**, 690–698 (1987).
21. Reid, M. J. & Menten, K. M. Radio photospheres of long-period variable stars. *Astrophys. J.* **476**, 327–346 (1997).
22. Kwok, S. Radiation pressure on grains as a mechanism for mass loss in red giants. *Astrophys. J.* **198**, 583–591 (1975).
23. Bester, M. *et al.* Measurement at 11 micron wavelength of the diameters of α Orionis and α Scorpii, and changes in effective temperature of α Orionis and very recent dust emission. *Astrophys. J.* **463**, 336–343 (1996).
24. Carilli, C. L., Holdaway, M. A. & Sowiński, K. P. *Fast Switching at the VLA* (VLA Scientific Memo. no. 169, National Radio Astronomical Observatory, Socorro, NM, 1996).

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Extreme oxygen-isotope compositions in magnetite from unequilibrated ordinary chondrites

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Primitive meteorites (such as the unequilibrated ordinary chondrites) have undergone only minor thermal processing on their parent asteroids, and thus provide relatively unaltered isotopic records from the early Solar System. For terrestrial materials, oxygen isotope compositions form a linear array called the terrestrial fractionation line¹. In meteorites the oxygen isotopic composition commonly deviates from this line², the magnitude of the deviation being expressed by the quantity $\Delta^{17}\text{O}$. Such deviations, which cannot be explained by mass-dependent fractionation processes, are probably caused by the mixing of two or more nebular components having different nucleosynthetic histories, for example, solids and gas. But no direct evidence for the oxygen isotopic composition of the latter (which is the dominant oxygen reservoir) has hitherto been available. Here we report *in situ* oxygen-isotope measurements of magnetite grains

in unequilibrated ordinary chondrites. Magnetite (which formed by aqueous alteration of metal in the parent asteroid) may serve as a proxy for nebular H_2O . We measured a value of $\Delta^{17}\text{O} \approx 5\text{‰}$, much higher than typical values of 0–2‰ in ordinary-chondrite silicate grains. Our results imply that a nebular component of high- $\Delta^{17}\text{O}$ H_2O was incorporated into the parent asteroid of the unequilibrated ordinary chondrites.

The discovery of large oxygen-isotope variations both within³ and among⁴ the chondrite groups demonstrated the existence of significant isotopic heterogeneities in the solar nebula. At the places and times in the nebula where the chondrite groups (or sets of related groups) formed, nebular grains incorporated material from reservoirs having distinct oxygen-isotope compositions reflecting different nucleosynthetic histories. An important factor is the unique cosmochemistry of oxygen that simultaneously makes it the dominant rock-forming element as well as one of the principal components of the nebular gas phase. For this reason, disequilibrium oxygen-isotope distributions among meteorites are commonly modelled in terms of two reservoirs, one solid, the other gaseous (see, for example, Clayton and Mayeda⁵).

In minerals from unequilibrated ordinary chondrites (UOCs), major cations such as Mg^{2+} and Fe^{2+} have not exchanged appreciably since formation in the nebula, and in most silicates, O diffuses more slowly than these ions^{6,7}. Thus most minerals retain their nebula compositions. However, fluid transported along grain surfaces in asteroids has produced some new, low-temperature minerals, for example magnetite⁸ and phyllosilicates⁹.

A minor subset of UOCs contain magnetite (Fe_3O_4)-carbide assemblages; Krot *et al.*⁸ inferred that the magnetite resulted from oxidation of Fe–Ni metal, probably by H_2O in an asteroid. Because all the oxygen in magnetite comes from the oxidant, the study of magnetite can constrain the O-isotope composition of the oxidizing fluid.

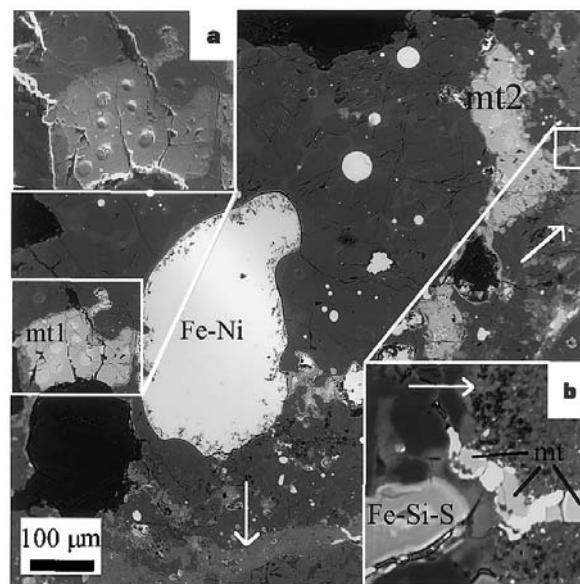


Figure 1 Back-scattered-electron image of part of low-FeO porphyritic olivine chondrule (PO1) in Semarkona USNM1805-5. Arrows indicate boundaries between the chondrule and matrix. In the chondrule white nodules are Fe–Ni metal with taenite, carbides and troilite. Light-grey objects with irregular shapes are magnetite (mt1 and mt2); the magnetite grains are surrounded by fayalite-like minerals $\sim 5\text{ }\mu\text{m}$ thick. Dark areas on top of the image and at bottom of mt1 are plucked regions. **a**, Scanning electron microscopy image of mt1, showing seven craters formed by ion-microprobe sputtering. **b**, High-magnification back-scattered-electron image of chondrule–matrix boundary. A magnetite-bearing vein extends from the chondrule into the matrix implying that this vein formed after the chondrule and matrix were compacted together.

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We used the UCLA Cameca ims 1270 ion microprobe to make *in situ* measurements of O-isotope abundances in magnetite and olivine from two UOCs, LL3.0 Semarkona (the most primitive chondrite¹⁰) and LL3.6 Ngawi (type 3.0 indicates the least altered chondrites¹¹; Ngawi mean subtype by A. Rubin, personal communication). Measurements were made at high mass-resolving power by using a defocused Cs⁺ beam to sputter ~12–25 μm diameter craters (Fig. 1a) on carbon-coated polished thin sections. We measured $^{16}\text{O}^-$ ions in a Faraday cup, $^{17}\text{O}^-$ and $^{18}\text{O}^-$ on an electron multiplier. A more detailed description of the techniques is given elsewhere¹².

We studied 11 magnetite grains in Semarkona and 2 in Ngawi; sizes ranged from ~30 to ~180 μm . Magnetite is relatively abundant in Semarkona; point-counting an area of ~2 cm^2 yields an abundance of 40 mg g^{-1} (corresponding to 11 mg g^{-1} oxygen). Whereas kamacite and taenite nodules always show smooth, rounded and embayed surfaces, magnetite grains have ragged boundaries (Fig. 1) suggesting irregular growth. Some magnetite assemblages show strong textural evidence of *in situ* formation; for example, Fig. 1b shows a magnetite–cohenite vein filling a crack in a chondrule and adjacent matrix, implying that the vein formed after the chondrule and matrix were compacted together in the parent asteroid.

Oxygen-isotope compositions of magnetite grains are summarized in Table 1 and plotted in Fig. 2. Compositions of chondrule olivines of Semarkona measured at UCLA during the same period are also shown in Fig. 2; as expected, these are similar to the compositions of UOC chondrules¹³. They will be discussed in detail elsewhere. Four generalizations may be made: (1) magnetite compositions in Semarkona and Ngawi are similar; (2) the Semarkona data show a range of ~13‰ in $\delta^{18}\text{O}$; (3) a regression line through the magnetite compositions has a slope of 0.70; and (4) the mean $\Delta^{17}\text{O}$ is ~5‰.

The mean $\Delta^{17}\text{O}$ of ~5‰ is the highest measured in solid

materials of Solar-System origin, although interstellar Al_2O_3 grains have more extreme compositions^{14,15}. Our $\Delta^{17}\text{O}$ values of Semarkona magnetite are somewhat higher than those reported by Saxton *et al.*¹⁶ (mean $\Delta^{17}\text{O}$ of ~3‰), but the data sets overlap within the uncertainties. Three measurements of two Ngawi magnetite grains plot close to the mean of Semarkona magnetite.

The $\delta^{18}\text{O}$ values in Semarkona magnetite span a wide range of ~13‰. Because there is the potential for such variations to be the result of sample contamination or a mass-dependent instrumental artefact¹⁷, we examined these possibilities in detail. The data were corrected for instrumental drift in the standard magnetite; corrections were typically small, at most 1.5‰ in $\delta^{18}\text{O}$. The sputtered craters were examined, but no ‘contaminating’ inclusions of silicates or other oxides were observed. Although some craters have cracks, there is no systematic difference between data for cracked and crack-free craters. If variable instrumental fractionation were due to grain-boundary (that is, topographic) effects, we would expect that measurements on smaller grains would show more scatter than larger grains. We observed the reverse; the largest magnetite, 1805mt1, yielded an isotopic variation of 10‰ in $\delta^{18}\text{O}$, and several smaller magnetites yielded much smaller ranges (Table 1 and Fig. 2). These arguments make it unlikely that the large variation is an experimental artefact.

There is no resolvable correlation between $\delta^{18}\text{O}$ and position within an individual magnetite grain. This is not inconsistent with expectations regarding mineral growth and scaling during corrosion. Unfortunately, it has the consequence that we are not able to specify whether $\delta^{18}\text{O}$ increased or decreased during the course of magnetite growth.

Formation of the magnetite from a semi-infinite H_2O reservoir (for example, the solar nebula) would yield relatively constant $\delta^{18}\text{O}$ because the fractionation factor between magnetite and water is not very temperature sensitive in the range of likely formation temperatures for magnetite in chondrites¹⁸. The large (13‰) range

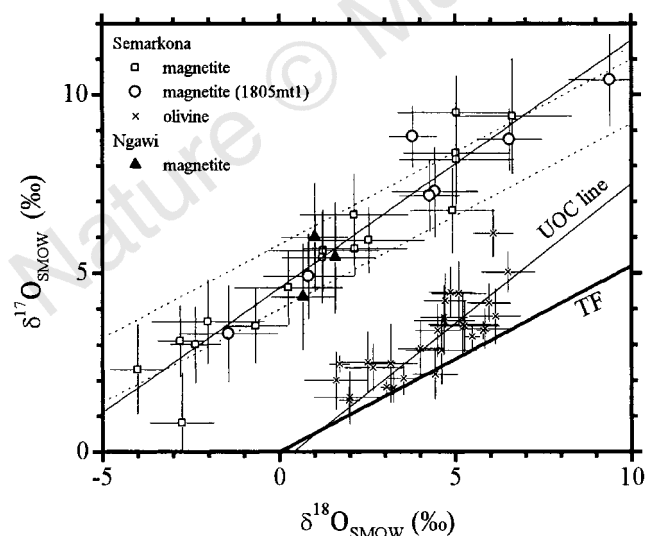


Figure 2 Oxygen-isotope measurements for Semarkona and Ngawi magnetite. Data are plotted on a $\delta^{18}\text{O}$ – $\delta^{17}\text{O}$ diagram; 1σ error bars are shown. Ion microprobe data for Semarkona olivine are also shown; these agree well with previous whole-rock and chondrule data, which fall near the unequilibrated ordinary chondrite (UOC) line. The magnetite $\Delta^{17}\text{O}$ values are ~4‰ larger than those of olivine. The terrestrial-fractionation (TF) line and a regression line for our magnetite data are shown for reference. The band bounded by dotted lines represents mass-dependent fractionation with a $\Delta^{17}\text{O}$ of $4.9 \pm 0.9\%$ (95% confidence interval for the mean of UOC magnetite $\Delta^{17}\text{O}$ values).

Table 1 Oxygen isotope compositions of Semarkona and Ngawi magnetites

Sample	$\delta^{18}\text{O} \pm 1\sigma^* \dagger$ (‰)	$\delta^{17}\text{O} \pm 1\sigma^* \dagger \ddagger$ (‰)	$\Delta^{17}\text{O} \S$ (‰)	Sitell
15 July 1996 Semarkona UCLA229				
1-1¶	-2.8 ± 0.6	3.1 ± 1.0	4.6	Matrix
2-1	-4.0 ± 0.8	2.3 ± 1.3	4.4	PP1
2-2	-2.4 ± 0.7	3.0 ± 1.1	4.3	PP1
14 October 1996 Semarkona USNM1805-5				
1-1	9.4 ± 1.2	10.4 ± 1.3	5.5	PO1
1-2	-1.4 ± 1.4	3.3 ± 1.3	4.1	PO1
1-3	4.4 ± 1.2	7.3 ± 1.3	5.0	PO1
1-4	0.8 ± 1.3	4.9 ± 1.2	4.5	PO1
2-1	4.9 ± 1.2	6.7 ± 1.2	4.2	PO1
2-2	5.0 ± 1.5	8.4 ± 1.5	5.8	PO1
2-3	6.6 ± 1.7	9.4 ± 1.6	6.0	PO1
3	1.2 ± 1.1	5.4 ± 1.3	4.8	Matrix
14 November 1996 Semarkona UNM312				
1	5.0 ± 1.7	9.5 ± 1.0	6.9	PP2
2	5.0 ± 1.6	8.2 ± 1.2	5.6	PP2
3-1	-2.0 ± 1.6	3.6 ± 1.2	4.7	Matrix
3-2	0.3 ± 1.5	4.6 ± 1.0	4.5	Matrix
3-3	2.1 ± 1.5	6.6 ± 1.1	5.5	Matrix
4¶	2.1 ± 1.5	5.7 ± 0.7	4.6	Rim
5-1	1.2 ± 1.6	5.6 ± 1.1	5.0	Matrix
5-2	2.5 ± 1.6	5.9 ± 0.9	4.6	Matrix
15 November 1996 Ngawi USNM2483				
1-1	1.0 ± 0.9	6.0 ± 1.5	5.5	Matrix
1-2	0.7 ± 0.9	4.3 ± 1.5	4.0	Matrix
2	1.6 ± 1.1	5.4 ± 1.5	4.6	Rim
17 November 1996 Semarkona USNM1805-5				
1-5	6.5 ± 0.9	8.8 ± 0.9	5.4	PO1
1-6	3.8 ± 0.7	8.8 ± 0.9	6.9	PO1
1-7	4.3 ± 0.9	7.2 ± 1.0	4.9	PO1
15 January 1997 Semarkona UCLA229				
1-2¶	-0.7 ± 0.9	3.5 ± 1.0	3.9	Matrix
3	-2.8 ± 0.9	0.8 ± 1.4	2.3	Rim

* $\delta^{18}\text{O} = \{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{SMOW}}] - 1\} \times 10^3$, where $\delta^{18}\text{O}$ is in units of per mil, SMOW indicates standard mean ocean water value and $\delta^{17}\text{O}$ is defined analogously.

† Errors include the standard deviations of the standard during each analytical session.

‡ OH^- interferences were $\leq 0.5\%$, typically $\leq 0.1\%$.

§ $\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.528\delta^{18}\text{O}$.

¶ PO, porphyritic olivine chondrule; PP, porphyritic pyroxene chondrule; PO1 and PP1 are low-FeO; PO2 is high-FeO; rim indicates rim around chondrules.

¶¶ Weighted means of two or three successive measurements on the same spot.

observed therefore suggests that a limited supply of oxidant was largely consumed during magnetite formation (resulting in Rayleigh-type fractionation). This reinforces the conclusion of Krot *et al.*⁸ that the magnetite formed in an asteroidal setting.

We therefore examined models involving asteroidal oxidation by H_2O . There are no clear constraints on the number of independent oxygen reservoirs in the ordinary-chondrite part of the solar nebula. In ordinary chondrites, mean whole-rock $\Delta^{17}O$ (ref. 13) correlates with degree of oxidation measured by bulk $FeO/(FeO + Mg)$ ratios¹⁹. Although it is possible that the oxygen in UOC magnetite is fully independent of the oxygen in silicates, we investigated models that could explain not only the high $\Delta^{17}O$ of magnetite but also the correlation between $\Delta^{17}O$ and degree of oxidation. We defined a UOC line having a slope of 0.78 (Fig. 2) based on O-isotope data for whole-rock falls and for chondrules¹³. According to this picture, the high- $\Delta^{17}O$ oxidant that formed UOC magnetite should have a composition on or near this UOC line.

We do not try to define the asteroidal source of the water. To produce the observed slope in the magnetite data (0.70) requires exchange between the oxidant and another reservoir having lower $\Delta^{17}O$; we suggest that UOC chondrule silicates are a plausible choice. The observed fractionation is produced by simultaneously forming magnetite and phyllosilicates, with the latter consuming 2.5 times as much oxygen as the former. This amount of phyllosilicate is somewhat higher than that obtained by a comparison of our estimate abundance (11 mg g^{-1}) of O in magnetite with the H_2O concentration of 16.2 mg g^{-1} ($= 14.4 \text{ mg g}^{-1} \text{ O}$) measured in Semarkona¹⁹, but some phyllosilicate water may have been lost during mild metamorphism. We assume that the H_2O was present as a liquid, that the equilibrium fractionation between magnetite and $H_2O(l)$ is -11‰ in $\delta^{18}O$ and that between phyllosilicate OH and $H_2O(l)$ is $+12\text{‰}$ (roughly corresponding to $T = 300 \text{ K}$ based on estimated bond-water O-isotope fractionations²⁰), and that, after formation, magnetite and phyllosilicate remained isolated

from the remaining H_2O . To account for a slope of 0.70, we further assume a starting $\Delta^{17}O$ of 6.6‰ and that, after each 0.5% of the $H_2O(l)$ reacted, 0.15% of the oxygen remaining in this phase was replaced by chondrule oxygen with $\delta^{18}O \approx 5.0\text{‰}$, $\delta^{17}O \approx 3.5\text{‰}$ (ref. 13).

The first magnetite to form has $\delta^{18}O = 10\text{‰}$, thus the $\delta^{18}O$ of the $H_2O(l)$ was 21‰ . As shown in Fig. 3, the magnetite oxygen tracks that of the remaining H_2O . The trend of the magnetite composition curves slightly but the mean slope is close to that of the data array, 0.70. This simple model of magnetite formation can account for the observed trend by interpreting the oxidant to have been H_2O with an O-isotope composition relatively close to the extrapolation of the UOC trend to $\Delta^{17}O \approx 7\text{‰}$. In principle, the model could be tested by analysing the O-isotope composition of the H_2O evolved from phyllosilicates at low temperatures; although the $\Delta^{17}O$ of this H_2O would include a substantial contribution from the anhydrous precursor, it should still have a $\Delta^{17}O$ appreciably higher than the bulk meteorite.

An interesting question is whether the nebular $H_2O(g)$ could have had such a high $\Delta^{17}O$ value while the silicate precursors of the chondrules were forming. Cosmochemical evidence seems best understood in terms of near-complete volatilization of pre-solar materials in the nebula²¹ followed by recondensation. If the $\Delta^{17}O$ of the nebular gas were $\geq 6\text{‰}$ when chondrule precursors were forming, it is remarkable that chondrule $\Delta^{17}O$ values are $0\text{--}2\text{‰}$. This might suggest that $\Delta^{17}O$ values in the UOC region were initially near 0‰ , but that subsequently infalling materials had higher $\Delta^{17}O$ values, and that the $\Delta^{17}O$ of the nebular gas gradually increased. □

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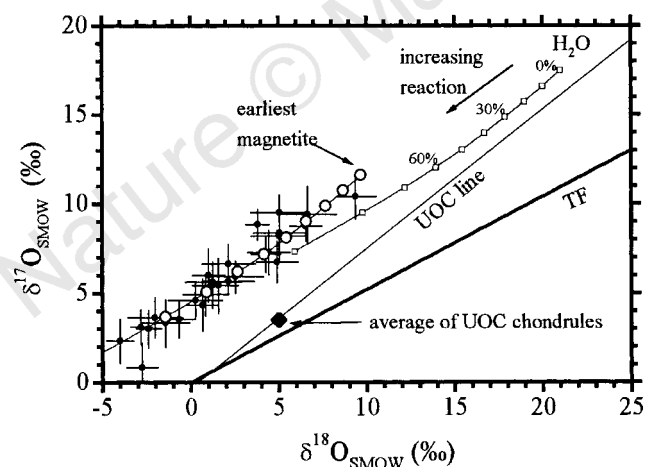


Figure 3 A model calculation for formation of magnetite and phyllosilicates. This was made to reproduce the O-isotope trend of Semarkona magnetite having a slope of 0.70. Initial $H_2O(l)$ was assumed to have $\delta^{18}O = 21.0$ and $\delta^{17}O = 17.5\text{‰}$ ($\Delta^{17}O = 6.6\text{‰}$). The equilibrium fractionation factors between magnetite and $H_2O(l)$ of -11‰ and between hydroxyl O in smectite and $H_2O(l)$ of $+12\text{‰}$ were used; points show increments of 10% in fraction of water reacted. It was assumed that 2.5 times as much as oxygen goes into forming phyllosilicates as into magnetite. To reproduce the trend of decreasing $\Delta^{17}O$ with decreasing $\delta^{18}O$, we assume that after each 0.5% fractionation, 0.15% of the $H_2O(l)$ oxygen is replaced with silicate oxygen having the composition of average UOC chondrules.

1. Clayton, R. N. Oxygen isotopes in meteorites. *Annu. Rev. Earth Planet. Sci.* **21**, 115–149 (1993).
2. Clayton, R. N., Onuma, N., Grossman, L. & Mayeda, T. K. Distribution of the presolar component in Allende and other carbonaceous chondrites. *Earth Planet. Sci. Lett.* **34**, 209–224 (1977).
3. Clayton, R. N., Grossman, L. & Mayeda, T. K. A component of primitive nuclear composition in carbonaceous meteorites. *Science* **182**, 485–488 (1973).
4. Clayton, R. N., Onuma, N. & Mayeda, T. K. A classification of meteorites based on oxygen isotopes. *Earth Planet. Sci. Lett.* **30**, 10–18 (1976).
5. Clayton, R. N. & Mayeda, T. K. The oxygen isotope record in Murchison and other carbonaceous chondrites. *Earth Planet. Sci. Lett.* **67**, 151–161 (1984).
6. Morioka, M. Cation diffusion in olivine, I. Cobalt and magnesium. *Geochim. Cosmochim. Acta* **44**, 759–762 (1980).
7. Ryerson, F. J., Durham, W. B., Cherniak, D. J. & Lanford, W. A. Oxygen diffusion in olivine: Effect of oxygen-fugacity and implications for creep. *J. Geophys. Res.* **44**, 4105–4118 (1989).
8. Krot, A. N. *et al.* Carbide-magnetite assemblages in type-3 ordinary chondrites. *Geochim. Cosmochim. Acta* **61**, 219–237 (1997).
9. Hutchison, R., Alexander, C. M. O. & Barber, D. J. The Semarkona meteorite; First recorded occurrence of smectite in an ordinary chondrite, and its implication. *Geochim. Cosmochim. Acta* **51**, 1875–1882 (1987).
10. Grossman, J. N. Semarkona: The least metamorphosed ordinary chondrite. (abstr.) *Meteoritics* **20**, 656–657 (1985).
11. Sears, D. W. G., Hasan, E. A., Batchelor, J. D. & Lu, J. Chemical and physical studies of type 3 chondrites—XI: Metamorphism, pairing, and brecciation of ordinary chondrites. *Proc. Lunar Planet. Sci. Conf.* **21**, 493–512 (1991).
12. Choi, B.-G., McKeegan, K. D., Leshin, L. A. & Wasson, J. T. Origin of magnetite in oxidized CV chondrites: in situ measurement of oxygen isotope compositions of magnetite and olivine in CV3 Allende. *Earth Planet. Sci. Lett.* **146**, 337–349 (1997).
13. Clayton, R. N., Mayeda, T. K., Goswami, J. N. & Olsen, E. J. Oxygen isotope studies of ordinary chondrites. *Geochim. Cosmochim. Acta* **55**, 2317–2337 (1991).
14. Huss, G. R., Fahey, A. J., Gallino, R. & Wasserburg, G. J. Oxygen isotopes in circumstellar Al_2O_3 grains from meteorites and stellar nucleosynthesis. *Astrophys. J.* **430**, L81–L84 (1994).
15. Nittler, L. R., Alexander, C. M. O'D., Gao, X., Walker, R. M. & Zinner, E. K. Interstellar oxide grains from the Tieschitz ordinary chondrite. *Nature* **370**, 443–446 (1994).
16. Saxton, J. M., Lyon, I. C., Turner, G. & Hutchison, R. Oxygen isotopes in Semarkona magnetite. (abstr.) *Meteoritics* **30**, 572–573 (1995).
17. Shimizu, N. & Hart, S. R. Isotope fractionation in secondary ion mass spectrometry. *J. Appl. Phys.* **53**, 1303–1311 (1982).
18. Rowe, M. W., Clayton, R. N. & Mayeda, T. K. Oxygen isotopes in separated components of CI and CM meteorites. *Geochim. Cosmochim. Acta* **58**, 5341–5347 (1994).
19. Jarosewich, E. Chemical analyses of meteorites: A compilation of stony and iron meteorite analyses. *Meteoritics* **25**, 323–337 (1990).
20. Savin, S. M. & Lee, M. Isotopic studies of phyllosilicates. *Rev. Mineral.* **19**, 189–223 (1988).
21. Palme, H. & Boynton, W. V. in *Protostars and Planets III* (eds Levy, E. & Lunine, J. I.) 979–1004 (Univ. Arizona Press, Tucson, 1993).

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Superconductivity at 25.5 K in electron-doped layered hafnium nitride

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The electronic properties of crystals with a layered structure can be radically altered by the intercalation, between the layers, of guest species that act as electron donors or acceptors. Such studies have been performed extensively on graphite, transition-metal dichalcogenides and oxide bronzes¹. Interest in redox intercalation reactions² has increased recently because the high-transition-temperature (high- T_c) superconductors based on copper oxide also have layered structures, the superconductivity occurring within two-dimensional CuO_2 planes separated by charge-reservoir oxide layers³. Similarly, in metal-doped fullerenes, which show relatively high transition temperatures, the electron donor atoms sit in the interstitial sites between adjacent fullerene balls⁴. In a previous study⁵, we described a layered nitride, $\beta\text{-ZrNCl}$, consisting of Zr–N double layers sandwiched between two close-packed chlorine layers. On lithium intercalation, the crystal changed from a semiconductor to a metal, and became a superconductor at 13 K. Here we report the properties of the isostructural compound $\beta\text{-HfNCl}$. After electron-doping the crystal by lithium intercalation, we observe superconductivity with a T_c of up to 25.5 K. This transition temperature is higher than that observed in any intermetallic compound, and suggests that layered nitride structures may offer transition temperatures comparable to those observed in layered copper oxide structures.

$\beta\text{-HfNCl}$ was prepared by the reaction of Hf metal powder with NH_4Cl under a stream of ammonia at 650°C , and purified by a chemical transport with the aid of NH_4Cl , according to the method reported^{6,7} for the preparation of $\beta\text{-ZrNCl}$. A 0.1 M lithium naphthalene solution (Naph.–Li) in tetrahydrofuran (THF) was prepared by dissolving an equimolar amount of naphthalene and lithium in THF which had been purified by distillation. Lithium intercalation was carried out by dispersing $\beta\text{-HfNCl}$ into the 0.1 M Naph.–Li solution for one day with the molar ratios of $\text{Li}/\text{HfNCl} = 0.2\text{--}2.0$, followed by washing with THF on a Teflon filter in an argon-filled glove box. The amount of lithium uptake was determined by an inductively coupled plasma spectrometer on the sample dissolved in a mixture of hydrofluoric acid and nitric acid. The X-ray powder diffraction (XRD) patterns of the intercalated samples were recorded under an argon atmosphere in a cylindrical cover with polyethylene windows by using graphite-monochromatized $\text{Cu K}\alpha$ radiation. The magnetic susceptibility was measured using a SQUID magnetometer (MPMS-5, Quantum Design, San Diego, USA). The electrical conductivity was measured by using a four-probe method on the powder sample compressed at a pressure of 1.8 GPa in an Ar filled glove box.

Layer-structure crystals of $\beta\text{-ZrNCl}$ were first prepared by Juza and Heners⁸, and the crystal structure was determined by Juza and Friedrichsen⁹. The structure can be related to the CdCl_2 type layer structure; the Zr–N–N–Zr layers occupy the Cd positions and are sandwiched between the close-packed chloride layers. The layers with a sequence of $[\text{Cl}\text{--Zr}\text{--N}\text{--N}\text{--Zr}\text{--Cl}]$ are stacked with each other by a weak van der Waals interaction as shown in Fig. 1. The structure is analogous to recently discovered layered metal-rich rare-earth carbide halides $\text{RE}_2\text{C}_2\text{X}_2$, where RE is Y or La, and X is Cl, Br or I, the metal-rich carbide layer RE_2C_2 being sandwiched

between halide layers¹⁰. The halides $\text{Y}_2\text{C}_2\text{I}_2$ and $\text{Y}_2\text{C}_2\text{Br}_2$ showed superconductivity at $T_c = 9.97\text{ K}$ and 5.04 K , respectively.

In a previous study⁵, lithium was intercalated into the van der Waals gap between the chloride layers of $\beta\text{-ZrNCl}$. $\beta\text{-ZrNCl}$ is isoelectronic with ZrS_2 , and is characterized¹¹ as a semiconductor with a bandgap of $\sim 3\text{ eV}$. On lithium intercalation, $\beta\text{-ZrNCl}$ is changed into a metallic compound with a colour change from pale yellow-green to black. The electrons are transferred from the intercalated lithium atoms to the ZrN layers through chlorine layers, and the empty t_{2g} band is partially filled with electrons, giving the metallic behaviour. The lithium-intercalated compound became a superconductor with a T_c of 13 K. The intercalated

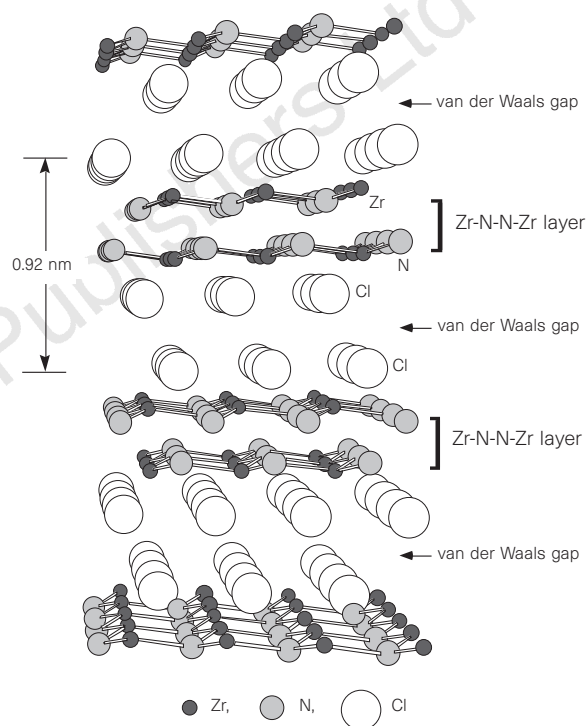


Figure 1 Structural model of $\beta\text{-ZrNCl}$ (ref. 5) (isostructural with $\beta\text{-HfNCl}$).

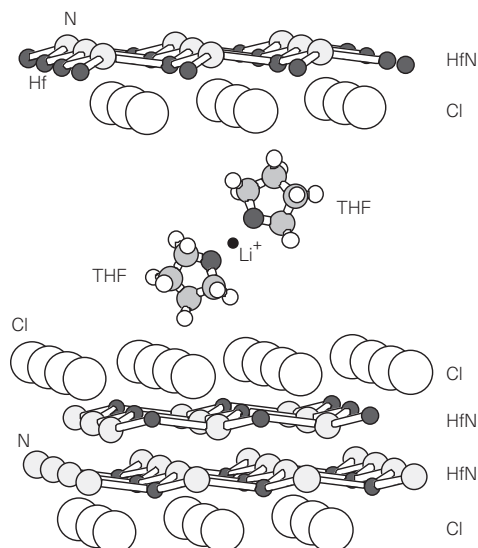


Figure 2 Schematic structural model of the arrangement of tetrahydrofuran (THF) co-intercalated with lithium between the HfNCl layers. The THF contents (γ) in $\text{Li}_x(\text{THF})_\gamma\text{HfNCl}$ were tentatively estimated to be in a range of 0.3–0.4 by analogy with the corresponding zirconium compounds obtained in the previous study¹⁶.

compound obtained by the reaction with *n*-butyllithium (*n*-BuLi) contained only lithium between the layers, and organic polar solvent molecules such as propylene carbonate and THF could be post-intercalated with lithium, expanding the basal spacing from 0.93 nm to >2 nm (ref. 12), while the superconducting behaviour was not influenced by the co-intercalation. These findings suggest that the superconductivity occurs within the thin two-dimensional ZrN layers separated by the close-packed chlorine layers. We note that this is, to our knowledge, the first layered nitride superconductor, although layered nickel boride containing nitrogen ($\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$) was reported¹³ to be superconducting at 13 K.

β -HfNCl is isostructural with β -ZrNCl. The structural para-

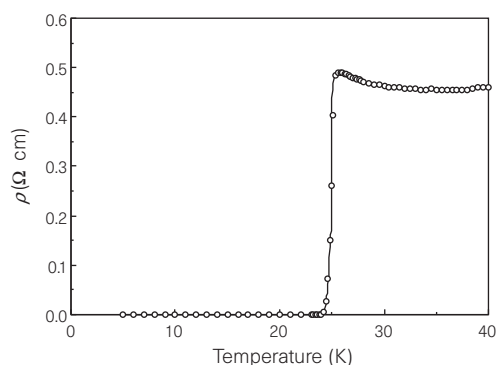


Figure 3 Electrical resistivity of $\text{Li}_{0.48}(\text{THF})_y\text{HfNCl}$ as a function of temperature.

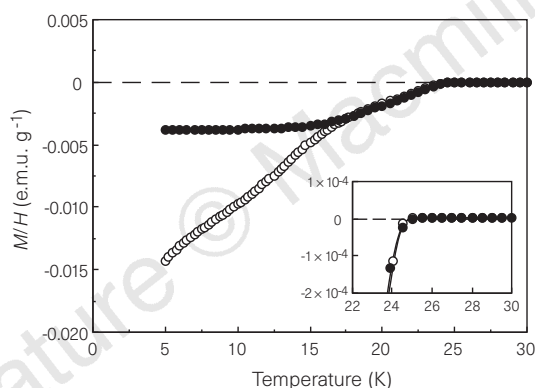


Figure 4 Magnetic susceptibility of $\text{Li}_{0.48}(\text{THF})_y\text{HfNCl}$. Open circles, zero field cooling; filled circles, field cooling. The susceptibility was recorded up to 30 K in a magnetic field of 50 Oe. The inset shows the onset of the superconducting transition at expanded scale.

Table 1 Structural parameters of the nitrides

	ZrNCl	HfNCl*	ZrN	HfN
Structure type	CdCl ₂ type		NaCl type	
Lattice const. (nm)				
<i>a</i>	0.3606†	0.3573	0.4576§	0.4525
<i>c</i> (3 × <i>d</i>)	2.767†	2.768		
Bond length (nm)				
M–N	0.2127	0.2108	0.2289	0.2263
M–M	0.3606	0.3573	0.3237	0.3200
Bond angle				
N–M–N	115.9°	115.9°	90°	90°
<i>T_c</i> (K)	13.0‡	25.5‡	10.7	8.8

* The composition was determined to be $\text{Hf}_{1.00}\text{N}_{1.00}\text{Cl}_{1.00}$ (found: Hf, 76.8; N, 6.0; Cl, 15.3%) by the chemical analysis method used previously⁹ for β -ZrNCl. The oxygen contamination was <0.04% after the chemical transport. The powder sample was single-phase by X-ray diffraction analysis. Atomic coordinates⁹ of β -ZrNCl were used for the calculation of a Hf–N bond distance and a N–Hf–N bond angle.

† Ref. 6.

‡ *T_c* for the lithium-intercalated phases.

§ Powder Diffraction File 35-753.

|| Powder Diffraction File 33-592

eters of the two kinds of layered nitrides are compared in Table 1. On lithium intercalation with Naph.-Li/THF, the colour of β -HfNCl crystals was also changed from white to black, and the XRD analysis showed that the basal plane spacing (*d*) increased, from 0.923 nm in the starting crystal to 1.40 and 1.87 nm, depending on the amount of lithium intercalated. The formation of by-products such as HfN and LiCl were not detected. The expanded spacings indicate the formation of co-intercalated phases of THF molecules with lithium between the layers. Co-intercalation compounds of layered chalcogenides and oxide bronzes have been reviewed by Schöllhorn¹⁴ and Johnston¹⁵. Different expanded spacings correspond to different types of orientations of THF molecules around the intercalated Li; single and double molecular layers for the 1.40- and 1.87-nm phases, respectively. The intercalated phases with different spacings co-existed; the larger-spacing phase was predominant for the samples with $x > 0.3$ in $\text{Li}_x(\text{THF})_y\text{HfNCl}$. Similar expanded spacings were also obtained in the reaction of β -ZrNCl with Naph.-Li/THF in our previous study¹⁶, and the inter-layer molecular arrangements were estimated on the basis of the one-dimensional electron distribution along the direction normal to the basal plane. Figure 2 shows a schematic structural model of the co-intercalated phase with a basal spacing of 1.87 nm.

Figure 3 shows the electrical resistivity of $\text{Li}_{0.48}(\text{THF})_y\text{HfNCl}$ (mainly composed of the 1.87-nm phase) as a function of temperature. The resistivity showed a sharp drop at 25.5 K and became zero at 24.5 K. The 10–90% width of the transition is 0.7 K. Figure 4 shows the magnetic susceptibility of the same sample measured by a SQUID magnetometer. The sample showed a superconducting transition due to the occurrence of the Meissner shielding at ~26 K. This temperature is in good agreement with the transition temperature obtained by resistivity measurement. The existence of the hysteresis between the two magnetization curves for the zero-field cooling (ZFC) and the field cooling (FC) modes indicates that the compound is a type-II superconductor. The volume susceptibility at 5 K was determined to be –0.065 on the basis of the ZFC data; the theoretical density of 4.3 g cm^{-3} was estimated from the XRD data. This value is ~80% of the theoretical value ($-1/4\pi$) for perfect diamagnetism, indicating that this is a bulk superconductivity.

It is well known that a series of transition-metal nitrides with the rock-salt structure become superconductors, such as TiN, ZrN, HfN and NbN with *T_c*s at 5.5, 10.7, 8.8 and 18.0 K, respectively^{17,18}. The structure of β -ZrNCl can be thought of as composed of ZrN double layers sliced from a ZrN crystal of the NaCl structure and sandwiched by chloride layers. As the *T_c* of HfN (8.8 K) is lower than that of ZrN (10.7 K), it was expected that β -HfNCl might have a lower *T_c* than that of β -ZrNCl, even though it might become a superconductor on lithium intercalation. It is surprising that the *T_c* of $\text{Li}_x(\text{THF})_y\text{HfNCl}$ is much higher than that of the zirconium analogue. It should be noted that the lattice dimensions of HfNCl are very close to those of ZrNCl. The M–N bond lengths in β -MNCl (where M is Zr, Hf) are shorter than those of the corresponding nitrides of the NaCl structure (Table 1); also, the hexagonal networks of ZrN and HfN between the chlorine layers are almost coplanar, with the bond angle N–M–N of 115.9° against 90° for the NaCl structure. The stronger bonding in the M–N network and the coplanarity may be the reason for the higher *T_c* of the layered phase compared with the NaCl phases. The superconductivity occurs within the electron-doped thin HfN double layers, which are separated from each other by chlorine layers with organic interlayers as shown in Fig. 2. It would be reasonable to expect an enhanced anisotropic effect in the superconductivity in the co-intercalated system. The co-intercalated system with the expanded spacings might provide a way to investigate the effect of dimensionality on the superconducting properties.

The amount of lithium uptake by β -HfNCl could be controlled by changing the amount of Naph.-Li used. All of the lithium-

intercalated samples showed bulk superconductivity. The T_c determined by magnetic susceptibility gradually decreased from 25.5 to 24.4 K with the increase of the amount of lithium intercalation from $x = 0.13$ to 0.97 in $\text{Li}_x(\text{THF})_y\text{HfNCl}$; mixtures of the two co-intercalated phases with basal spacings of 1.40 and 1.87 nm were observed for $x < 0.3$, and the 1.87-nm phase was mainly obtained for $x > 0.3$. Similar decreases of T_c by the overdoping of alkali metals were also observed on the intercalated series of $\beta\text{-ZrNCl}$ with alkali metals¹². The decrease of T_c can be explained in terms of the increase of the screening of the phonon–electron interaction, which may decrease the average electron-pairing interaction for the formation of Cooper pairs. Although the reason for the high T_c of $\text{Li}_x(\text{THF})_y\text{HfNCl}$ is not clear, our recent study (unpublished data) shows that $\beta\text{-ZrNCl}$ and $\beta\text{-HfNCl}$ can form continuous solid solutions over the whole compositional range, and the T_c of the lithium intercalation compounds increases linearly with the increase of the amount of substitution of Zr with Hf. This finding should be a clue to solving the mechanism of the high- T_c superconductivity of this system. □

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- Whittingham, M. S. & Jacobson, A. J. (eds) *Intercalation Chemistry* (Academic, New York, 1982).
- Dresselhaus, M. S. New directions in intercalation research. *Mol. Cryst. Liq. Cryst.* **244**, 1–12 (1994).
- Cava, R. J. Structural chemistry and the local charge picture of copper oxide superconductors. *Science* **247**, 656–662 (1990).
- Taniigaki, K. Development of superconductors based on C_{60} . *Springer Proc. Phys.* **81**, 172–185 (1996).
- Yamanaka, S., Kawaji, H., Hotehama, K. & Ohashi, M. A new layer-structured nitride superconductor. Lithium-intercalated β -zirconium nitride chloride, Li_xZrNCl . *Adv. Mater.* **9**, 771–774 (1996).
- Ohashi, M., Yamanaka, S., Sumihara, M. & Hattori, M. Novel synthesis of the layer structured $\beta\text{-ZrNCl}$ by the direct reaction of zirconium metal or zirconium hydride with ammonium chloride. *J. Solid State Chem.* **75**, 99–104 (1988).
- Ohashi, M., Yamanaka, S. & Hattori, M. Chemical vapor transport of layer structured crystal $\beta\text{-ZrNCl}$. *J. Solid State Chem.* **77**, 342–347 (1988).
- Juza, R. & Heners, J. Über Nitridhalogenide des Titans und Zirkons. *Z. Anorg. Allg. Chem.* **332**, 159–172 (1964).
- Juza, R. & Friedrichsen, H. Die Kristallstruktur von $\beta\text{-ZrNCl}$ und $\beta\text{-ZrNBr}$. *Z. Anorg. Allg. Chem.* **332**, 173–178 (1964).
- Henn, R. W., Schnelle, W., Kremer, R. K. & Simon, A. Bulk superconductivity at 10 K in the layered compounds $\text{Y}_2\text{C}_2\text{I}_2$ and $\text{Y}_2\text{C}_2\text{Br}_2$. *Phys. Rev. Lett.* **77**, 374–377 (1996).
- Ohashi, M., Yamanaka, S. & Hattori, M. Preparation and electrical conductivities of layer structured crystals ZrNX ($\text{X}=\text{Br}, \text{I}$). *J. Ceram. Soc. Jpn Int. Edn* **97**, 1175–1181 (1989).
- Kawaji, H., Hotehama, K. & Yamanaka, S. Superconductivity of alkali metal intercalated β -zirconium nitride chloride A_xZrNCl ($\text{A}=\text{Li}, \text{Na}, \text{K}$). *Chem. Mater.* **9**, 2127–2130 (1997).
- Zandbergen, H. W., Jansen, J., Cava, R. J., Krajewski, J. J. & Peck, W. F. Jr Structure of the 13-K superconductor $\text{La}_2\text{Ni}_2\text{B}_2\text{N}_3$ and the related phase LaNiB_2N . *Nature* **372**, 759–761 (1994).
- Schöllhorn, R. in *Intercalation Chemistry* (eds Whittingham, M. S. & Jacobson, A. J.) 315–360 (Academic, New York, 1982).
- Johnston, D. C. Observation of new cointercalation compounds in the system $\text{Na}_{0.35}(\text{H}_2\text{O})_y\text{TaS}_2$ ($0 \leq y \leq 2$) from powder X-ray diffraction measurements. *J. Less-Common Met.* **84**, 327–347 (1982).
- Ohashi, M., Uyeoka, K., Yamanaka, S. & Hattori, M. Co-intercalation of tetrahydrofuran and propylene carbonate with alkali metals in $\beta\text{-ZrNCl}$ layer structured crystal. *Bull. Chem. Soc. Jpn* **64**, 2814–2818 (1991).
- Roberts, B. W. Survey of superconductive materials and critical evaluation of selected properties. *J. Phys. Chem. Ref. Data* **5**, 581–795 (1976).
- Pessall, N. & Hulm, J. K. Superconducting alloys of interstitial compounds. *Physics* **2**, 311–328 (1966).

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Structures of medium-sized silicon clusters

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Silicon is the most important semiconducting material in the microelectronics industry. If current miniaturization trends continue, minimum device features will soon approach the size of atomic clusters. In this size regime, the structure and properties of materials often differ dramatically from those of the bulk. An

enormous effort has been devoted to determining the structures of free silicon clusters^{1–22}. Although progress has been made for Si_n with $n < 8$, theoretical predictions for larger clusters are contradictory^{2–22} and none enjoy any compelling experimental support. Here we report geometries calculated for medium-sized silicon clusters using an unbiased global search with a genetic algorithm. Ion mobilities²³ determined for these geometries by trajectory calculations are in excellent agreement with the values that we measure experimentally. The cluster geometries that we obtain do not correspond to fragments of the bulk. For $n = 12$ –18 they are built on a structural motif consisting of a stack of Si_4 tricapped trigonal prisms. For $n \geq 19$, our calculations predict that near-spherical cage structures become the most stable. The transition to these more spherical geometries occurs in the measured mobilities for slightly larger clusters than in the calculations, possibly because of entropic effects.

The structures of small ($n < 8$) silicon clusters have been extensively studied by theoretical methods. Except for $n = 5$, the structures of these clusters have been confirmed by anion photoelectron spectroscopy²⁴, or by Raman²⁵ and infrared²⁶ measurements on matrix-isolated clusters. As the cluster size increases, it becomes much more difficult to find the lowest-energy structure in theoretical studies because the number of possible geometries increases exponentially. Extensive searches have been performed for slightly larger silicon clusters ($n = 8$ –11) but some of the conclusions are still the subject of debate^{6,7,9,10,20}. For $n > 13$, only geometries constructed following assumed bonding schemes have been presented. None of the structures proposed for $n > 7$ have been experimentally verified. Anion photoelectron spectroscopy and Raman and infrared studies of matrix-isolated clusters have not yet been able to provide ground-state vibrational frequencies for clusters with more than seven atoms. This leaves ion mobility measurements as the only experimental method currently available to provide information about the structures of silicon clusters containing over a dozen atoms. This method has been successfully applied to carbon clusters²⁷. Previous mobility measurements²³ for Si_n^+ have only been interpreted qualitatively to indicate that silicon clusters become prolate between $n = 10$ and 23, then for larger clusters a structural transition to a more spherical geometry occurs. Here we take advantage of the recent implementation of a classical trajectory method for evaluating mobilities²⁸, and perform a quantitative comparison of the measured mobilities with those determined for the calculated geometries.

An unbiased global search for the ground state structures of

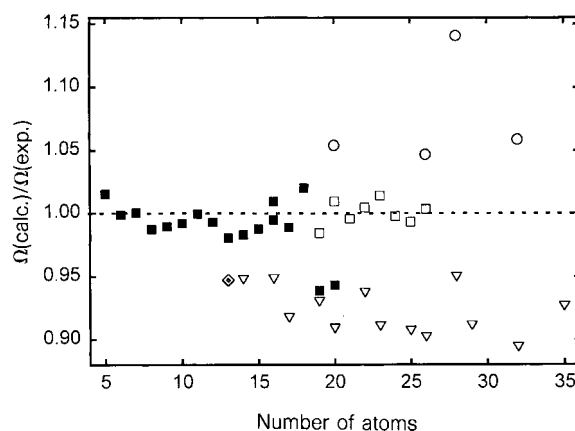


Figure 1 Relative deviations of the collision integrals for proposed silicon cluster geometries from the values measured at 298 K. Empty circles and triangles are for the families of “puckered six-fold rings”^{18,19} and “alternating triangles”²⁰. Filled and empty squares are for our geometries described in the text, and shown in Figs 2 and 3. Icosahedral Si_{13} is shown by a diamond. For $n < 18$, measurements were also performed at 78 K and the relative deviations are similar to those shown here.

silicon clusters with 11–20 atoms was performed by means of a genetic algorithm²⁹. This is an optimization strategy based on modelling biological evolution. A population of structures is optimized, and the fittest members selected to ‘parent’ the next generation through a ‘mating’ process. Here we operate with a population of 16 clusters with four ‘parents’. The ‘offspring’ clusters of each generation are relaxed by molecular dynamics quenching. The fully relaxed structure is incorporated into the parent population if its energy is below that of its parents, and discarded otherwise. The procedure is continued for at least 600 generations, with multiple ecologies ensuring a truly global search. Initially, the energies are estimated using a new tight-binding potential (C.Z.W. *et al.*, manuscript in preparation) developed to reproduce the results of density functional theory calculations using the local density approximation (LDA). For the promising isomers, LDA was used to evaluate the energies. To check the convergence of the search procedure, a parallel independent search was performed using simulated annealing with a Car-Parrinello LDA technique. For $n \leq 16$, simulated annealing and the genetic algorithm find the same global minima. However, for larger clusters, the energies of structures found by the genetic algorithm are lower. The searches were performed for neutral clusters, but the lowest-energy isomer for each size was fully relaxed as a cation. The changes in the geometries are negligible in all cases.

Ion mobilities were measured using a tandem quadrupole drift-tube apparatus that has previously been described in detail³⁰. The clusters were generated by laser vaporization of a silicon rod, entrained in a helium flow, ionized by a 1.1 keV electron beam, mass selected, and injected into a drift tube containing helium buffer gas. Mobilities are measured by injecting a 20–50 μ s pulse of cluster ions into the drift tube and recording the arrival time distribution at the detector. Measurements were performed at drift-tube temperatures of 78 and 298 K. The drift-tube buffer gas pressure was 2.5–9.4 torr, the drift voltage was 13.3 V cm^{-1} , and the injection energy was 50 eV. In all cases, the experiments were carried out in the low drift field regime where the mobility is independent of the field.

The reduced mobility of a gas phase ion is inversely proportional to the orientationally averaged collision integral for the ion and buffer gas. Collision integrals for candidate geometries were calculated by propagating classical trajectories in a realistic intermolecular potential²⁸. This potential is constructed as a sum of pairwise Si–He interactions plus a charge-induced dipole term. Each pairwise interaction is treated as having the Lennard–Jones form and the charge is assumed to be delocalized over all the atoms. Recent work for carbon clusters³¹ has demonstrated that this model is more accurate than previously used approximations. To apply this model here, we first need to determine the elementary Si–He potential. This is accomplished by fitting the temperature dependence of the mobility of a cluster with known geometry. The two largest clusters with experimentally confirmed geometries, Si₆ (D_{4h} tetragonal) and Si₇ (D_{3h} pentagonal bipyramid)^{25,26} were used. The only parameters adjusted in the fit are the depth of the potential, ϵ , and the intercept, σ . The values obtained were $\epsilon = 1.35$ meV and $\sigma = 3.50$ Å. Mobilities calculated for the correct Si_{*n*} structures should agree with the measurements at both 78 and 298 K to within²⁷ ~2%. This error margin includes the experimental uncertainty and the estimated uncertainties in both the mobility calculations and the bond lengths and bond angles of the candidate structures.

We have evaluated the mobilities for two isomer families proposed in the literature: the “stacked puckered six-fold rings” of Kaxiras and Jackson^{18,19} and the “stacked triangles” of Grossman and Mitas²⁰. The results are shown in Fig. 1. The mobilities calculated for both isomer families deviate systematically from the measurements for the prolate isomers: the “stacked six-fold rings” are always too prolate and the “stacked triangles” are not prolate

enough. The mobilities calculated for the lowest-energy geometries resulting from our unbiased structural optimization (filled squares in Fig. 1) are within 2% of the measurements for $n \leq 18$. The LDA energies of these geometries are substantially lower than for all previously proposed isomers for $n > 11$.

For $n = 8$ the measured mobilities compare well with those evaluated for the previously accepted lowest-energy geometry^{7,8,15}: a C_{2h} distorted bicapped octahedron. In all-electron calculations⁷, the C_s distorted tricapped octahedron and C_{2v} distorted tricapped trigonal prism (TTP) are nearly degenerate for $n = 9$, and the T_d tetracapped octahedron and C_{3v} tetracapped trigonal prism are nearly degenerate for $n = 10$. Recently, Chelikowsky and collaborators⁶ have found a C_{2v} capped stacked rhombi structure to be the ground state of Si₉. The mobilities calculated for all three Si₉ isomers agree with the measurements. However, the mobility computed for T_d Si₁₀ is in significantly worse agreement with the measurements than the C_{3v} geometry, which virtually rules out the T_d geometry. This supports recent calculations²⁰ affirming the C_{3v} geometry as the ground state for Si₁₀. We can definitely eliminate all non-compact (tetrahedral bonding network) structures and unreconstructed fragments of bulk Si lattice advanced for small silicon clusters¹⁰.

For $n = 11$, we obtain the C_{2v} structure of Rohlfing and Raghavachari⁸ as the global minimum. However, the other six isomers considered in ref. 8—except for the two pentacapped octahedrons that are high in energy—have mobilities which also agree with our measured values. The previously accepted structures for $n = 12$ and 13 were a C_{2v} hexacapped trigonal prism¹⁶ and a C_{3v} capped trigonal antiprism²¹, respectively. Our calculated geometries, which are shown in Fig. 2, are lower in energy by 0.6 eV for Si₁₂ and 0.3 eV for Si₁₃. Unfortunately, for these clusters the old and new

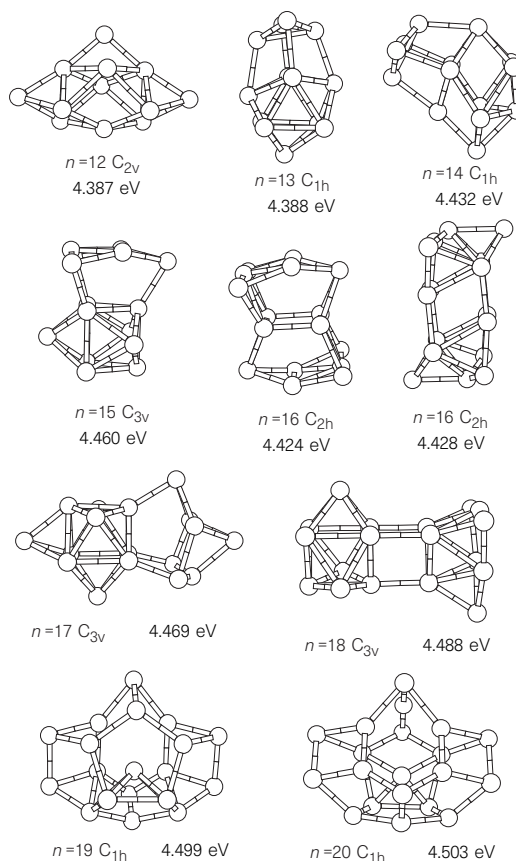


Figure 2 The LDA global minima for the Si_{*n*} ($n = 12$ –20) neutrals. Cohesive energies per atom are indicated. The two geometries shown for Si₁₆ are essentially degenerate.

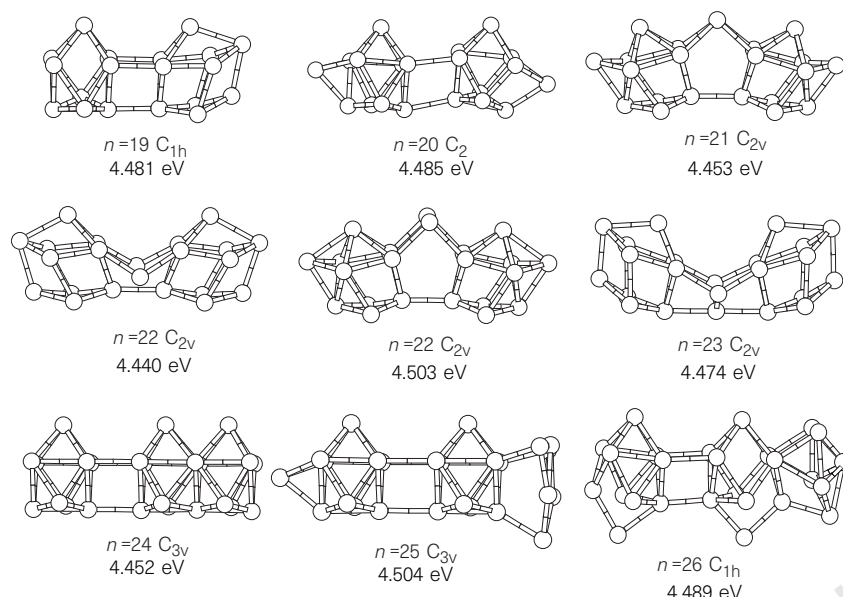


Figure 3 Prolate geometries for $n = 19$ – 26 constructed by stacking Si_9 TTP subunits. The structures for $n = 19$ and 20 were relaxed, while the others are not necessarily local minima.

structures could not be distinguished experimentally as their mobilities are within 1% of each other. We can, however, exclude icosahedral Si_{13} , the existence of which has been the subject of much debate^{4,5,13,14,17,20,21} (see Fig. 1). The mobility becomes really sensitive to geometry for $n \geq 14$. In this size range, our structures are lower in energy than those previously known²⁰ by 1.2 eV (Si_{14}), 0.9 eV (Si_{16}) and 2.7 eV (Si_{17}), and are the only proposed geometries to have the right mobilities. For $n = 15$ and 16 , we have found two structures that are degenerate within the accuracy of the LDA calculation. For $n = 15$, only the C_{3v} structure has a calculated mobility that agrees with the measurements, whereas for $n = 16$ the calculated mobilities of both geometries agree. In the $n < 19$ size range, our geometries can be visualized as stacked Si_9 tricapped trigonal prisms (see Fig. 2) as first conjectured by Jarrold and Constant²³.

Starting from $n = 19$, our computed global minima drastically change from the prolate assemblies of Si_9 subunits to more compact cage-like geometries (Fig. 2). The calculated mobilities for this new family of isomers disagree with the measured values until $n \geq 24$, where they fit the second experimentally observed ("spherical") isomer. So the structural rearrangement shown in Fig. 2 apparently corresponds to the "prolate-to-spherical" transition that begins at $n = 24$ in the experiments²³. The discrepancy in the point of onset could be due to an entropic effect similar to that found for carbon clusters. For carbon, the fullerene is predicted to be the most stable geometry³² for a cluster as small as C_{20}^+ , but C_{30}^+ is the smallest fullerene observed experimentally²⁷. This occurs because at the high temperature required to induce isomerization, the fullerene is not a low-free-energy structure for the smaller clusters. It only becomes competitive³³ around C_{28} . Because the prolate structures are not the lowest-energy isomers for silicon clusters with $n \geq 19$ (according to the LDA calculations), geometries for the prolate isomers that persist in the mobility measurements to larger cluster sizes²³ cannot be obtained using the genetic algorithm. However, these geometries can be built-up by stacking Si_9 TTP prisms following the patterns established for $n < 19$. We have constructed such structures up to $n = 26$ (see Fig. 3). Their mobilities (empty squares in Fig. 1) agree with the measurements, which supports our assignment of all prolate clusters as Si_9 TTP stacks. These structures for $n > 19$ are not necessarily unique, as several geometries with minor variations often differ in mobility by less than 1%, for example two isomers for $n = 22$ in Fig. 3. As these geometries are not the result of a systematic search, it is possible that other, lower-energy structures might also fit the mobilities of the "prolate" family for $n \geq 19$. However, our prolate isomers are lower in energy than any pre-

viously proposed^{18–20,22} for Si_n , $n = 19$ – 26 , by 1–4 eV. In fact, they are (to our knowledge) the first geometries reported for $n = 17, 18$ and $n = 22$ – 26 that are energetically stable against spontaneous fission into two smaller clusters.

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- Pacchioni, G. & Koutecky, J. Silicon and germanium clusters. A theoretical study of their electronic structures and properties. *J. Chem. Phys.* **84**, 3301–3310 (1986).
- Tomanek, D. & Schluter, M. A. Calculation of magic numbers and the stability of small Si clusters. *Phys. Rev. Lett.* **56**, 1055–1058 (1986).
- Slee, T., Zhenyang, L. & Mingo, D. M. P. Polyhedral skeletal electron pair theory of bare clusters. 1. Small silicon clusters. *Inorg. Chem.* **28**, 2256–2261 (1989).
- Chelikowsky, J. R., Glassford, K. M. & Phillips, J. C. Interatomic force fields for silicon microclusters. *Phys. Rev. B* **44**, 1538–1545 (1991).
- Phillips, J. C. Electron-correlation energies and the structure of Si_3 . *Phys. Rev. B* **47**, 14132–14135 (1993).
- Vasiliev, I., Ögüt, S. & Chelikowsky, J. R. *Ab Initio* calculations for the polarizabilities of small semiconductor clusters. *Phys. Rev. Lett.* **78**, 4805–4808 (1997).
- Raghavachari, K. & Rohlfing, C. M. Bonding and stabilities of small silicon clusters: a theoretical study of Si_7 – Si_{10} . *J. Chem. Phys.* **89**, 2219–2234 (1988).
- Rohlfing, C. M. & Raghavachari, K. A theoretical study of small silicon clusters using an effective core potential. *Chem. Phys. Lett.* **167**, 559–565 (1990).
- Raghavachari, K. & Rohlfing, C. M. Structures of Si_{10} . Are there conventionally bonded low-energy isomers? *Chem. Phys. Lett.* **198**, 521–525 (1992).
- Messmer, R. P. & Patterson, C. H. Long bonds in silicon clusters: a failure of conventional Møller-Plesset perturbation theory? *Chem. Phys. Lett.* **192**, 277–282 (1992).
- Mistriotis, A. D., Froudakis, G. E., Vondras, P. & Flytzanis, N. Model potential for silicon clusters and surfaces. *Phys. Rev. B* **47**, 10648–10653 (1993).
- Jug, K. & Krack, M. Consistent parameterization of semiempirical MO methods. *Int. J. Quant. Chem.* **44**, 517–531 (1992).
- Gu, B., Li, Z. & Zhu, J. Electrical structure of small icosahedral silicon clusters. *J. Phys.: Condens. Matter* **5**, 5255–5260 (1993).
- Gong, X. G., Zheng, Q. Q. & He, Y. Z. Structural properties of silicon clusters: an empirical potential study. *J. Phys.: Condens. Matter* **7**, 577–584 (1995).
- Menon, M. & Subbaswamy, K. Nonorthogonal tight-binding molecular-dynamics scheme for silicon with improved transferability. *Phys. Rev. B* **55**, 9231–9234 (1996).
- Ramakrishna, M. V. & Bahel, A. Combined tight-binding and density functional molecular dynamics investigation of Si_{12} cluster structure. *J. Chem. Phys.* **104**, 9833–9840 (1996).
- Wales, D. J. Electronic structure of small silicon clusters. *Phys. Rev. A* **49**, 2195–2198 (1994).
- Kaxiras, E. & Jackson, K. Shape of small silicon clusters. *Phys. Rev. Lett.* **71**, 727–730 (1993); Structural models for intermediate-sized Si clusters. *Z. Phys. D* **26**, 346–348 (1993).
- Pederson, M. R., Jackson, K., Porezag, D. V., Hajnal, Z. & Frauenheim, T. Vibrational signatures for low-energy intermediate-sized Si clusters. *Phys. Rev. B* **54**, 2863–2867 (1996).
- Grossman, J. C. & Mitas, L. Quantum Monte Carlo determination of electronic and structural properties of Si_n clusters. *Phys. Rev. Lett.* **74**, 1323–1326 (1995); Family of low-energy elongated Si_n ($n \leq 50$) clusters. *Phys. Rev. B* **52**, 16735–16738 (1995).
- Rothlisberger, U., Andreoni, W. & Giannozzi, P. Thirteen-atom clusters: equilibrium geometries, structural transformations, and trends in Na, Mg, Al, and Si. *J. Chem. Phys.* **96**, 1248–1256 (1992).
- Song, J., Ulloa, S. E. & Drabold, D. A. Soliton-induced lattice relaxation and the electronic and vibrational spectra of silicon clusters. *Phys. Rev. B* **53**, 8042–8051 (1996).
- Jarrold, M. F. & Constant, V. A. Silicon cluster ions: evidence for a structural transition. *Phys. Rev. Lett.* **67**, 2994–2997 (1991).
- Arnold, C. C. & Neumark, D. M. Study of Si_4 and Si_4^+ using threshold photodetachment (ZEKE) spectroscopy. *J. Chem. Phys.* **99**, 3353–3362 (1993).
- Honea, E. C. *et al.* Raman spectra of size-selected silicon clusters and comparison with calculated structures. *Nature* **366**, 42–44 (1993).
- Li, S., Van Zee, R. J., Weltner Jr, W. & Raghavachari, K. Si_3 – Si_7 . Experimental and theoretical infrared spectra. *Chem. Phys. Lett.* **243**, 275–280 (1995).
- Von Helden, G., Hsu, M. T., Gotts, N. G. & Bowers, M. T. Carbon cluster cations with up to 84 atoms: structures, formation mechanism, and reactivity. *J. Phys. Chem.* **97**, 8182–8192 (1993).

28. Mesleh, M. F., Hunter, J. M., Shvartsburg, A. A., Schatz, G. C. & Jarrold, M. F. Structural information from ion mobility measurements: effects of the long-range potential. *J. Phys. Chem.* **100**, 16082–16086 (1996).
29. Deaven, D. M. & Ho, K. M. Molecular geometry optimization with a genetic algorithm. *Phys. Rev. Lett.* **75**, 288–291 (1995).
30. Jarrold, M. F. Drift tube studies of atomic clusters. *J. Phys. Chem.* **99**, 11–21 (1995).
31. Shvartsburg, A. A., Hudgins, R. R., Dugourd, Ph. & Jarrold, M. F. Structural elucidation of fullerene dimers using high-resolution ion mobility measurements and trajectory calculation simulations. *J. Phys. Chem. A* **101**, 1684–1688 (1997).
32. Bylaska, E. J., Taylor, P. R., Kawai, R. & Weare, J. H. LDA predictions of C_{20} isomerizations: neutral and charged species. *J. Phys. Chem.* **100**, 6966–6972 (1996).
33. Martin, J. M. L. C_{28} : the smallest stable fullerene? *Chem. Phys. Lett.* **255**, 1–6 (1996).

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Interactive effects of ozone depletion and vertical mixing on photosynthesis of Antarctic phytoplankton

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Photosynthesis of Antarctic phytoplankton is inhibited by ambient ultraviolet (UV) radiation during incubations^{1–4}, and the inhibition is worse in regions beneath the Antarctic ozone 'hole'⁴. But to evaluate such effects, experimental results on, and existing models of, photosynthesis^{5–7} cannot be extrapolated directly to the conditions of the open waters of the Antarctic because vertical mixing of phytoplankton alters UV exposure and has significant effects on the integrated inhibition through the water column^{2,8,9}. Here we present a model of UV-influenced photosynthesis in the presence of vertical mixing, which we constrain with comprehensive measurements from the Weddell-Scotia Confluence during the austral spring of 1993. Our calculations of photosynthesis integrated through the water column (denoted P_T) show that photosynthesis is strongly inhibited by near-surface UV radiation. This inhibition can be either enhanced or decreased by vertical mixing, depending on the depth of the mixed layer. Predicted inhibition is most severe when mixing is rapid, extending to the lower part of the photic zone. Our analysis reveals that an abrupt 50% reduction in stratospheric ozone could, in the worst case, lower P_T by as much as 8.5%. However, stronger influences on inhibition can come from realistic changes in vertical mixing (maximum effect on P_T of about $\pm 37\%$), measured differences in the sensitivity of phytoplankton to UV radiation ($\pm 46\%$) and cloudiness ($\pm 15\%$).

When progressively shorter wavelengths of UV are transmitted to samples of phytoplankton, photosynthesis is inhibited in a response that can be described as a function of wavelength^{1,2,7,8,10,11}. Results have been used to assess the influence of ozone depletion (which increases the short-wavelength UV-B, 290–320 nm) on primary productivity^{2,6,12}. However, simulated vertical mixing of natural samples from the Antarctic showed that inhibition of photosynthesis by UV radiation can be modulated by the temporal pattern of exposure². Because vertical mixing strongly influences open waters

of the Antarctic^{13,14}, models describing inhibition during variable exposure to UV radiation must be developed to quantify the effects of UV and ozone depletion on Antarctic photosynthesis.

An effect of mixing on inhibition is consistent with a response that depends on the history of UV exposure during the day, rather than on instantaneous UV irradiance¹⁵. Because little is known about the kinetics of inhibition by UV in open waters, we measured the time-course of photosynthesis under combinations of UV radiation and photosynthetically active radiation (PAR; 400–700 nm) for phytoplankton in the Weddell-Scotia Confluence (WSC) and showed that inhibition progressed during exposures to constant irradiance consistent with an exponential survival curve⁹, unlike diatoms in culture which quickly reached a stable level of inhibition¹⁵. Steady-state inhibition in the culture represented a balance between damage and repair processes¹⁵; in contrast the WSC results implied an absence of repair. Complementary experiments confirmed that recovery processes were insignificantly slow over the timescale of 0.5–3.5 h (ref. 9).

We constructed a model of photosynthesis in mixing surface layers of the WSC to evaluate how this dependence of UV inhibition on cumulative exposure affects P_T . Uninhibited photosynthesis, P_{T0} , is calculated for comparison. Previous models using irradiance-dependent inhibition suggested that the effects of ozone depletion

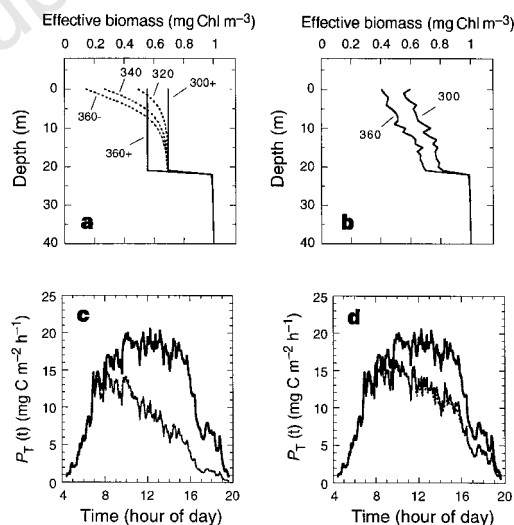


Figure 1 Models of photoinhibition during vertical mixing. **a**, The single-layer model: complete vertical mixing of photosynthetically effective biomass, B_{eff} (mg Chl m⁻³) to depth z_{mix} is generated at intervals τ_{mix} . During the intervening period, the water is static and B_{eff} declines as a function of cumulative exposure. This example is for $t = 300$ min until 360 min (– and + are before and immediately after 'mixing') on a typical cloudy day (31 October 1993): $z_{mix} = 21$ m, $\tau_{mix} = 1$ h, $O_3 = 300$ DU and B_{eff} initially is 1 mg Chl m⁻³. B_{eff} for the mixed layer declined to 0.56 from 0.69 mg Chl m⁻³. The BWF for 3 November was used: on that day, the mixed layer depth was 70 m, so this model predicts the effects of a shoaling in the mixed layer. **b**, A more conventional but computationally intensive lagrangian model of vertical mixing was used to calculate P (equation (1)) for individual particles. Although the distributions with depth seem much more realistic than in the single-layer model, the change in average mixed-layer B_{eff} , to 0.56 from 0.70 mg Chl m⁻³ (300–360 min), is almost identical. **c**, The agreement between models extends to the time-course of water-column photosynthetic rate (mg C m⁻² h⁻¹). The upper curve is uninhibited photosynthesis (irradiance-dependent, hence insensitive to vertical mixing), and the two lower curves (nearly indistinguishable) are the single-layer model (dotted line) and the lagrangian model (solid line). **d**, Water column photosynthetic rate for the single-layer model (lower solid line) compared with the three-layer model (lower dotted line), in which mixing rate decreases with depth (see Methods). This result, for 150 DU, $\tau_{mix} = 1$ h in a 90-m mixed layer, shows that the time course of P_T is insensitive to the assumption of uniform versus decreasing K_z with depth¹⁷, and that P_T is greater for deeper mixed layers.

are small: one model predicted that a 50% depletion would cause a reduction of <1% in P_T for the Southern Ocean south of 50° S⁶; the reduction is <5% in another model for the Bellingshausen Sea¹². Both models are by definition insensitive to vertical mixing.

Here, we represent inhibition as a decrease in photosynthetically competent biomass (B_{eff} , in units of mg Chl m⁻³), which in turn decreases photosynthesis (P , in units of mg C m⁻³ h⁻¹; see equation (1) in Methods). At specified time steps, the upper layer (depth $\leq z_{\text{mix}}$) is 'mixed' by calculating average B_{eff} in the layer, which serves as the initial biomass for the next interval (Fig. 1a). The influence of mixing rate was examined by changing the time step, τ_{mix} (0.16 to 16 h). This idealized representation of vertical mixing over a single layer was validated by comparison to a lagrangian model (Fig. 1b, c; see refs 16, 17) and to a three-layer model in which mixing time increased with depth (Figs 1d and 2b; see refs 17, 18). Using the first model, we examined the influences on P_T of the following: ozone depletion, observed variations in the sensitivity of phytoplankton to UV, depth and rate of vertical mixing, and typical cloudiness versus clear skies.

The analysis revealed complex interactions between UV radiation and vertical mixing. Consistent with experiments using simulated vertical circulation of Antarctic phytoplankton in bottles², mixing within the photic zone ($z_{\text{mix}} \leq 32$ m, depth of 1% surface photosynthetically usable radiation) caused increased inhibition (defined as $1 - (P_T/P_{\text{Tpot}})$) as compared to the static case (Fig. 2a, b). A harmful effect of vertical mixing has been suggested previously on the basis of experimental results on Antarctic phytoplankton^{2,19}, but mechanistic explanations could not be developed from available information. Here, the negative effect of mixing is the consequence

of the exponential decline of photosynthetic competence as a function of cumulative exposure (H_{inh} , dimensionless; see equation (1b) in Methods). For severe near-surface inhibition in the static case ($H_{\text{inh}}^* = 4$ for exposure symmetrical about midday), inhibition ($1 - (P/P_{\text{pot}})$; see ref. 9) is 0.76 for the full day, as observed for some incubations in containers^{19,20}. However, 75% of the inhibition (0.57/0.76) occurs during the first half of the day. In the afternoon under these static conditions, surface phytoplankton are already strongly inhibited, so decrements of B_{eff} hence P , are smaller. Vertical mixing brings deeper, less inhibited phytoplankton to the surface and more photosynthetic biomass is inactivated through the day. Also, inhibited phytoplankton are transported to the lower photic zone where light-limited rates are reduced as compared to the static case. Consequently, P_T is lower when the water is mixed within the photic zone. The process is analogous to models of the destruction of pollutants²¹ or viruses²² by UV radiation because, for the phytoplankton we studied, inhibition is a function of cumulative exposure, essentially irreversible for the timescale we consider (<1 d).

In contrast, mixing well below the photic zone lessens inhibition (Fig. 2a). In the model, deep vertical mixing maintains high B_{eff} at the surface, hence high rates of inactivation ($\Delta B_{\text{eff}}/\Delta t$). Although reductions of integrated B_{eff} (mg Chl m⁻²) are greatest for deep vertical mixing, the damage is distributed over a greater depth, so changes in B_{eff} (mg Chl m⁻³) within the photic zone are small (compare ref. 8). Simply, deep mixing has a net positive effect on P_T because deep phytoplankton represent a large reservoir of photosynthetic competence that would not contribute to P_T if it were not for vertical mixing. This compensation effect is less pronounced when mixing time increases with depth (Fig. 2b) so that residence time below the photic zone is longer. In the extreme, slow mixing is equivalent to no mixing, so both the negative and positive influences of vertical mixing are stronger for shorter mixing timescales in the model (Fig. 2a, b).

The depth and rate of vertical mixing influence not only overall inhibition, but also the incremental effects of changes in stratospheric ozone. For one example (Fig. 2c), predicted P_T for static water under severe O₃ depletion (150 Dobson units, DU) was 1.5% lower than under more normal 300 DU. Incremental inhibition (defined as $1 - P_T(150 \text{ DU})/P_T(300 \text{ DU})$) was maximal (5.9%) for rapid mixing to 30 m, and was less for shallower, deeper or slower mixing.

Measured sensitivity to UV radiation, quantified by biological weighting functions (BWFs; see equation (2) in Methods), varied by more than a factor of three between phytoplankton assemblages in the WSC⁹, strongly affecting water-column inhibition. For an example presented here (Fig. 2d), differences in sensitivity to UV were responsible for about a threefold range in modelled inhibition, regardless of mixing regime. This large variation demonstrates that one BWF cannot describe responses of Antarctic phytoplankton to UV^{6,7}, even if the spectral slopes of BWFs are similar¹⁰. Physiological variability also altered the interaction between vertical mixing and O₃ depletion, although subtly. Incremental inhibition associated with 50% O₃ depletion (range bars in Fig. 2d) was generally small (<10%), but higher for the assemblages that were less sensitive to UV. This is in part a consequence of the nonlinear dependence of inhibition on cumulative exposure (equation (1b) in Methods): effects of incremental H_{inh}^* are less when H_{inh} is already large.

A sensitivity analysis, constrained by direct measurements in the WSC, allows us to compare the relative influences of O₃ depletion, physiological variability, hydrographical variability and cloudiness on primary productivity in open waters of the Antarctic (Table 1). The range of modelled conditions is generally consistent with what we encountered in the WSC, with the exception of mixed layers <20 m and the extremes of O₃ concentration. Results indicate that O₃ depletion has a consistently negative effect on primary production during the spring. For the worst combination of BWF, mixing depth and mixing time, P_T was reduced by 6.2% (cloudy) to 8.5%

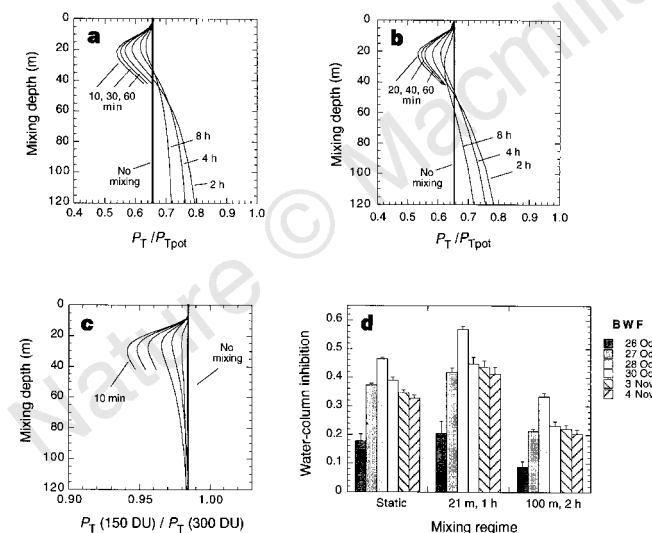


Figure 2 Model results constrained by direct measurements from the WSC⁹. **a**, Interactive effects of mixing depth and mixing timescale (curves labelled with τ_{mix}) on daily water-column productivity relative to the uninhibited rate, using the biological weighting function (BWF) of 3 November 1993, O₃ = 300 DU. Langmuir circulation can mix the upper water column rapidly, so mixing times ≤ 1 h are modelled for mixing depths ≤ 42 m. **b**, As in **a**, except for the three-layer mixing model (Fig. 1d). Nominal times are τ_{mix} for the upper two layers. **c**, Interactive effects of O₃ depletion (150 DU versus 300 DU) and mixing time as a function of mixing depth: proportional change in P_T for the same curves as in **a**. **d**, Water-column inhibition defined as $(1 - P_T/P_{\text{Tpot}})$; 300 DU) evaluated with the six BWFs from the WSC under three different conditions: no mixing, mixing to 21 m ($\tau_{\text{mix}} = 1$ h), and mixing to 100 m ($\tau_{\text{mix}} = 2$ h). Range bars indicate the enhancement of inhibition (relative to P_{Tpot} for 300 DU) associated with 50% O₃ depletion. The no-mixing example approximates results for day-long fixed-depth incubations⁴; results for shallow mixing are more relevant for the first three BWFs (sampled from mixed layers of about 30, 35 and 25 m); and the results for deep mixing better represent the latter three BWFs (from mixed layers of about 100, 70 and 120 m).

(clear sky) by 50% O₃ depletion, approximately the greatest depletion encountered during the cruise. For most modelled conditions, however, the reduction of P_T was a few per cent in response to 50% O₃ depletion. Measured variability in the sensitivity of phytoplankton to UV radiation (that is, BWFs) had a much greater influence; it is responsible for a range of as much as $\pm 46\%$ (more commonly near $\pm 25\%$) in P_T under a particular regime of irradiance and mixing. Similar strong influences on P_T are associated with vertical mixing. Although there is an interaction between mixing rate and mixing depth (Fig. 2), the extreme positive and negative effects ($+31\%$ and -44%) of mixing are associated with deep and shallow mixing, respectively, at the shortest mixing times. The influence of vertical mixing is less for longer mixing times (Fig. 2a) and for other combinations of BWF and O₃ depletion, yet generally greater than the effects of O₃ depletion. Typical cloudy conditions, as compared to clear skies, had a variable, though mostly negative influence on P_T . Decreased PAR would reduce P_{Tpot} by 24–27%; however, inhibition also decreases under cloudy skies, so the net effect of clouds on P_T ranged from -24% to $+5.7\%$, depending on vertical mixing and the sensitivity of phytoplankton to UV.

Our analysis differs in several respects from previous attempts to quantify photoinhibition in Antarctic phytoplankton: (1) BWFs were determined on the same assemblages for which P_T is modelled; (2) the dependence of photoinhibition on irradiance was explicitly rejected through experimentation and an appropriate model was developed; (3) the effects of vertical mixing (mixing rate and mixing depth) on P_T are quantified; and (4) results for typical cloudy conditions are compared with those for a clear sky. Still, two fundamental results of the analysis were consistent with previous findings^{2,4,12}: inhibition of P_T by UV radiation under normal O₃ conditions was very significant (often $>20\%$), and O₃ depletion made it worse, although only by a few per cent. Because estimates of UV effects on Antarctic phytoplankton are generally based in one way or another on fixed-depth incubations, it is encouraging that our results for static conditions compare well to what has been published: simulations of vertical mixing (Fig. 2) illustrate how

fixed-depth incubations can yield biased results (see also ref. 2).

We are confident that the results of our analysis describe the fundamental relationships between vertical mixing, solar radiation, and physiological responses of WSC phytoplankton to UV radiation, yet our results must be treated with great caution. First, it should be recognized that for each BWF, inhibition by UV can be accurately assessed only for the set of conditions on the day of sampling. The sensitivity analysis predicts what would happen if mixing, cloudiness or O₃ changed abruptly. For example, if a sensitive assemblage from a deep mixed layer or an uplifted stratum is trapped in a shallow mixed layer (as seems to have occurred on 28 October during the WSC cruise; ref. 9), severe inhibition should ensue (Fig. 2d). By the next day, acclimatization²³ as well as differential survival is likely to engender an assemblage more tolerant to UV, like those sampled from relatively shallow mixed layers⁹. Thus the largest inhibitions in our sensitivity analysis would represent possibly rare, transient events.

It should also be remembered that our model of inhibition (equation (1)), dependent on cumulative exposure and irreversible, was developed on the basis of short-term (0.5–3.5 h) experiments on phytoplankton from cold, deeply mixed surface layers⁹. It is possible that the feeble ability to repair damage to photosynthetic systems is the consequence of very low rates of respiration and growth in these light-limited phytoplankton (see ref. 14). If recovery could proceed on timescales of several hours to a day, some patterns in our data would be tempered, but not eliminated. We know that short-term irreversibility of inhibition does not hold for temperate phytoplankton in culture¹⁵ or for Antarctic phytoplankton from more protected waters⁷ where mean irradiance is probably higher and where repair mechanisms may be more active. Clearly, models should be modified as more information on the kinetics of inhibition and recovery in Antarctic waters becomes available.

Although the average effects in Table 1 reflect somewhat the choices that were made for inclusion in the sensitivity analysis, the ranges represent natural variability in Antarctic waters. We conclude that ozone depletion can inhibit primary productivity in open

Table 1 Influences on water-column productivity

Source of variability	Measure of variability	Per cent change					
		Strongest effect		Weakest effect		Average	
		Cloud	Clear	Cloud	Clear	Cloud	Clear
Ozone depletion*	$\frac{P_T(150 \text{ DU}) - P_T(300 \text{ DU})}{P_T(300 \text{ DU})}$	-6.2	-8.5	-0.8	-0.7	-2.7	-2.8
Physiological variability†	$P_T(P_{Tpot}(300 \text{ DU}))$	± 36.1	± 45.5	± 13.9	± 17.3	± 24.5	± 28.0
Depth of vertical mixing	$\frac{P_T(z_{mix}) - P_T(\text{static})}{P_T(\text{static})}$	-32.2 +30.9	-43.8 +29.7			-16.5 +24.8	-27.3 +21.4
Effect of cloudiness on inhibitions‡	$(P_T/P_{Tpot})[\text{cloud}] - (P_T/P_{Tpot})[\text{clear}]$	+14.3		+0.7		+6.2	
Effect of cloudiness on P_{Tpot}	$\frac{P_{Tpot}(\text{cloud}) - P_{Tpot}(\text{clear})}{P_{Tpot}(\text{clear})}$	-26.7		-23.5		-24.9	
Net effect of cloudiness	$\frac{P_T(\text{cloud}) - P_T(\text{clear})}{P_T(\text{clear})}$	-24.3§		+5.7¶		-16.1	

Data are the results of a sensitivity analysis using a model of water-column productivity constrained by direct measurements in the Weddell–Scotia Confluence. Per cent changes are presented for a typical cloudy day (31 October 1993) and for clear skies. Averages are very rough measures of central tendency, because they are influenced by the choice of conditions for the sensitivity analysis.

* The potential effect of an abrupt, 50% reduction in stratospheric ozone (see ref. 4) is represented as the per cent reduction of P_T ($\text{mg C m}^{-2} \text{ d}^{-1}$) for 150 DU as compared to 300 DU. The strongest and weakest effects are the extremes for all combination of conditions (6 BWFs, 21 z_{mix} and 10 τ_{mix}). Mixing times ≤ 1 h are not considered for $z_{mix} > 42$ m.

† Effects of physiological variability are quantified as the range of $P_T/P_{Tpot}(300 \text{ DU})$ for the six measured BWFs, normalized to the mean value for the six, evaluated for each combination of nine O₃ concentrations, 21 z_{mix} and 10 τ_{mix} . Results are expressed as $\pm$ half this range. Productivity was normalized to P_{Tpot} to focus on variability associated with differences in sensitivity to UV. P_{Tpot} is evaluated at 300 DU so that variations in the small increase of P_{Tpot} associated with O₃ depletion⁶ are considered.

‡ Extreme per cent reductions or enhancements associated with mixing for all combinations of BWF, z_{mix} and O₃. As z_{mix} and τ_{mix} are varied (Fig. 2a) the reduction of P_T is always greatest for the most rapid mixing ($\tau_{mix} = 10$ min) to an intermediate depth and maximum enhancement is for the most rapid, deepest mixing ($\tau_{mix} = 1.5$ h, $z_{mix} = 120$ m). The average effects of mixing depth are reported as means of the maximum reductions and enhancements for the 54 combinations of BWF and O₃ concentration. Effects approach zero as mixing rate slows.

§ Effects of cloudy conditions as compared to clear skies for all combinations of O₃, BWF, z_{mix} and τ_{mix} . The combinations of conditions associated with the strongest and weakest reductions of inhibition are not the same as those for the strongest and weakest reductions of P_{Tpot} .

¶ The greatest reduction of P_T associated with cloudiness.

‡ The greatest enhancement of P_T associated with cloudiness.

waters of the Antarctic, but that natural variability in exposures of phytoplankton to UV radiation, associated with vertical mixing and cloud cover, has an important role in either enhancing or diminishing the effect on water-column photosynthesis. Vertical mixing and UV radiation interact both directly in their effects on photosynthesis, and indirectly by influencing acclimatization and selection of phytoplankton. It should be recognized, however, that regardless of these natural interactions, UV radiation presents a significant environmental stress, and its effects are enhanced by ozone depletion. □

Methods

Calculation of water-column photosynthesis. The bio-optical model of Arrigo⁶, which incorporated our laboratory-derived model of PAR-dependent photosynthesis as inhibited by UV radiation¹¹, was modified to include our field-based biological model and time courses of UV and PAR exposure for given rates and depths of vertical mixing as represented by three alternative physical models.

Biological model. Photosynthesis, P ($\text{mg C m}^{-3} \text{ h}^{-1}$), is a saturating function of the irradiance absorbed by photosynthetic systems (photosynthetically usable radiation²⁴, E_{PUR} , $\mu\text{mol m}^{-2} \text{ s}^{-1}$), relative to a saturating value, E_k ($\mu\text{mol m}^{-2} \text{ s}^{-1}$):

$$P = P_s^{\text{eff}} (1 - e^{-E_{\text{PUR}}/E_k}) B_{\text{eff}} \quad (1a)$$

$$B_{\text{eff}}(T) = B_{\text{eff}}(0) e^{-H_{\text{inh}}^*} \quad (1b)$$

P is constrained by P_s^{eff} ($\text{mg C (mg Chl)}^{-1} \text{ h}^{-1}$), the maximum potential rate normalized to photosynthetically effective chlorophyll biomass, B_{eff} (mg Chl m^{-3}). Inhibition is represented as exponential inactivation of B_{eff} as a function of biologically effective exposure, H_{inh}^* (dimensionless). This definition simplifies the calculation of inhibition during mixing; we are not assuming destruction of pigment. H_{inh}^* is evaluated using a biological weighting function (BWF)²⁵, a set of weightings ($\epsilon_H(\lambda)$, reciprocal $\mu\text{mol m}^{-2}$) that quantify effectiveness of spectral exposure ($E(\lambda)$, $\mu\text{mol m}^{-2} \text{ s}^{-1} \text{ nm}^{-1}$), integrated over time, T) as a function of wavelength:

$$H_{\text{inh}}^* = \int_0^T \left(\sum_{\lambda=280 \text{ nm}}^{700 \text{ nm}} \epsilon_H(\lambda) E(\lambda) \Delta\lambda \right) dt \quad (2)$$

Uninhibited photosynthesis, P_{pot} , is calculated by setting $H_{\text{inh}}^* = 0$. Photosynthetic parameters in equation (1) and BWFs for equation (2) were experimentally determined on six occasions during October and November 1993⁹.

Physical model: single-layer mixing.

Step a.

$$\left\{ \begin{array}{l} E_{\text{dd}}(\lambda, 0^+, t) \\ E_{\text{ds}}(\lambda, 0^+, t) \end{array} \right\} \xrightarrow{\rho, \rho', \bar{\mu}_d} E(\lambda, 0^-, t) \xrightarrow{K(\lambda)} E(\lambda, z, t)$$

Step b.

$$\begin{array}{ccc} & \bar{a}_{\text{ps}}(\lambda) & \\ E(\lambda, z, t) & \xrightarrow{\quad} & E_{\text{PUR}}(z, t) \\ & \epsilon_H(\lambda) & \\ & \xrightarrow{\quad} & H_{\text{inh}}^*(z, t) \end{array}$$

Step c.

$$\begin{array}{ccc} H_{\text{inh}}^*(z, t) & \xrightarrow{\quad} & B_{\text{eff}}(z, t) \quad [z_{\text{mix}} \leq z \leq 120 \text{ m}] \\ B_{\text{eff}}(t = 0) & \xrightarrow{H_{\text{inh}}^*(z, t), \tau_{\text{mix}}, f(z_{\text{mix}})} & B_{\text{eff}}(z, t) \quad [0 \leq z \leq z_{\text{mix}}] \end{array}$$

Step d.

$$P_T = \sum_{z=0}^{120 \text{ m}} \sum_{t=0}^{16 \text{ h}} P(E_{\text{PUR}}(z, t), B_{\text{eff}}(z, t)) \Delta t \Delta z$$

Step a. A clear-sky solar radiation model²⁶, with enhanced spectral resolution in the UV⁶ is used to calculate the direct (E_{dd}) and diffuse (E_{ds}) components of downwelling spectral irradiance just above the surface ($E(\lambda, 0^+, t)$; $\mu\text{mol m}^{-2} \text{ s}^{-1} \text{ nm}^{-1}$), consistent with local meteorology (59.6° S, 52.1° W, 31 October 1993); column O_3 is varied in a sensitivity analysis. Attenuation by clouds, from the ratio of shipboard pyranometer readings to clear-sky predictions (cloud index,

CI) for this typical cloudy day, is considered to be spectrally neutral¹²; the resulting increase of E_{ds} as the expense of E_{dd} is calculated from an empirical relationship between CI and direct versus global shortwave irradiance²⁷ for each model time-step (Δt) of 5 min. Spectral scalar irradiance just below the surface, $E(\lambda, 0^-, t)$, is obtained by accounting for surface reflection of E_{dd} and E_{ds} (ρ and ρ'), refraction, and the contribution of direct and diffuse components to $\bar{\mu}_d$, the average cosine of the downwelling radiance distribution²⁸; upwelling irradiance was ignored. A profile of downwelling irradiance in four UV-wavebands (PUV-510, Biospherical Instruments) was used to estimate diffuse attenuation coefficients ($K(\lambda)$, m^{-1}), from 290 to 380 nm, with linear interpolation and extrapolation of $K(\lambda)$ versus λ . Estimates of spectral attenuation in the visible (LiCor submersible spectroradiometer) were merged to estimate $K(\lambda)$ from 290 to 700 nm, and $E(\lambda, z, t)$ was estimated as $E(\lambda, 0^-, t) \exp(-K(\lambda) \cdot z)$. **Step b.** $E(\lambda, z, t)$ is weighted by a normalized fluorescence excitation spectrum, $\bar{a}_{\text{ps}}(\lambda)$, for a diatom²⁹ to calculate $E_{\text{PUR}}(z, t)$ (ref. 24) for equation (1a); $H_{\text{inh}}^*(z, t)$ comes from equation (2), using $E(\lambda, z, t)$ and directly measured BWFs, $\epsilon_H(\lambda)$, for WSC phytoplankton⁹. **Step c.** Effective biomass, $B_{\text{eff}}(z, t)$, is evaluated for each time step (equation (1b)) using BWF results (equation (2)). At dawn ($t = 0$), B_{eff} is constant with depth, consistent with measurements on discrete samples within mixed layers in the study region. For stratified water ($z_{\text{mix}} \leq z \leq 120 \text{ m}$), $B_{\text{eff}}(z, t)$ depends only on H_{inh}^* at depth z from dawn through time t . Under the influence of mixing ($0 \leq z \leq z_{\text{mix}}$), water is static for the mixing period, τ_{mix} (h); before the next time-step, $B_{\text{eff}}(z)$ is instantaneously and evenly redistributed in the mixed layer, retaining the same average concentration (Fig. 1a). **Step d.** Photosynthesis, $P(z, t)$ ($\text{mg C m}^{-3} \text{ h}^{-1}$) is calculated from $E_{\text{PUR}}(z, t)$ and $B_{\text{eff}}(z, t)$ using P_s^{eff} and E_k from the same BWF experiments (equation (1a)). Daily photosynthesis integrated through the water column, P_T ($\text{mg C m}^{-2} \text{ d}^{-1}$), is calculated by numerical integration through time and over depth, and uninhibited P_{TPot} ($H_{\text{inh}}^* = 0$) is calculated for reference.

Lagrangian mixing model. Irradiance was specified using steps a and b, above. Then lagrangian trajectories were specified for 2,000 particles during simulated mixing from winds of 10 m s^{-1} (ref. 16, after ref. 17) corresponding to a timescale for vertical movement ($z_{\text{mix}}/(\text{modelled turbulent r.m.s. velocity})$)³⁰ of $\sim 0.6 \text{ h}$ for a mixed layer of 21 m. $E(\lambda, z, t)$ along the trajectory was used to evaluate P (equation (1)) for each particle. Because changes of mixing rate with depth in the mixed layer cannot be characterized robustly for this region, the coefficient of eddy diffusion K_z was assumed to be constant through the mixed layer, instead of declining with depth as in the original model¹⁷.

Three-layer mixing. To simulate decreased mixing with depth in the surface layer (see ref. 18), mixing is like the single-layer model, except that the mixed layer is divided in three and mixing occurs at intervals of $0.5 \tau_{\text{mix}}$ in the upper third of the mixed layer, τ_{mix} for the upper two layers, and $2.0 \tau_{\text{mix}}$ for all three layers.

Sensitivity analysis. The analysis was run using the single-layer model: for each of six sets of BWF parameters determined on phytoplankton from the WSC; under both cloudy and clear-sky conditions; varying z_{mix} from 0 to 120 m ($N = 21$); τ_{mix} from 0.16 to 16 h ($N = 10$); and O_3 from 75 to 450 DU ($N = 9$). A less comprehensive analysis was run with the three-layer model, yielding similar results. Mixing rates were not measured in the rough, windy WSC during our study (mean wind speed, 14.6 m s^{-1} ; mixed layer depths, 25–120 m during the October–November 1993 cruise), so we modelled a range of timescales that probably encompass conditions in open waters of the Antarctic^{13,30}. Mixing times $\leq 1 \text{ h}$ are not considered for $z_{\text{mix}} > 42 \text{ m}$.

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- Lubin, D. et al. A contribution toward understanding the biospherical significance of Antarctic ozone depletion. *J. Geophys. Res.* **97**, 7817–7828 (1992).
- Helbling, E. W., Villafañe, V. & Holm-Hansen, O. in *Ultraviolet Radiation in Antarctica: Measurements and Biological Effects* (eds Weiler, C. S. & Penhale, P. A.) 207–227 (Am. Geophys. Union, Washington DC, 1994).
- Vernet, M., Brody, E. A., Holm-Hansen, O. & Mitchell, B. G. in *Ultraviolet Radiation in Antarctica: Measurements and Biological Effects* (eds Weiler, C. S. & Penhale, P. A.) 143–158 (Am. Geophys. Union, Washington DC, 1994).
- Smith, R. C. et al. Ozone depletion: Ultraviolet radiation and phytoplankton biology in Antarctic waters. *Science* **255**, 952–959 (1992).
- Neale, P. J., Lesser, M. P. & Cullen, J. J. in *Ultraviolet Radiation in Antarctica: Measurements and Biological Effects* (eds Weiler, C. S. & Penhale, P. A.) 125–142 (Am. Geophys. Union, Washington DC, 1994).
- Arrigo, K. R. Impact of ozone depletion on phytoplankton growth in the Southern Ocean: large-scale spatial and temporal variability. *Mar. Ecol. Prog. Ser.* **114**, 1–12 (1994).
- Boucher, N. & Prézélin, B. B. An *in situ* biological weighting function for UV inhibition of phytoplankton carbon fixation in the Southern Ocean. *Mar. Ecol. Prog. Ser.* **144**, 223–236 (1996).

8. Smith, R. C. & Baker, K. S. in *The Role of Solar Ultraviolet Radiation in Marine Ecosystems* (ed. Calkins, J.) 509–537 (Plenum, New York, 1982).
9. Neale, P. J., Cullen, J. J. & Davis, R. F. Inhibition of marine photosynthesis by ultraviolet radiation: Variable sensitivity of phytoplankton in the Weddell-Scotia Confluence during the austral spring. *Limnol. Oceanogr.* (in the press).
10. Behrenfeld, M. J., Chapman, J. W., Hardy, J. T. & Lee, H. II Is there a common response to ultraviolet-B radiation by marine phytoplankton. *Mar. Ecol. Prog. Ser.* **102**, 59–68 (1993).
11. Cullen, J. J., Neale, P. J. & Lesser, M. P. Biological weighting function for the inhibition of phytoplankton photosynthesis by ultraviolet radiation. *Science* **258**, 646–650 (1992).
12. Boucher, N. P. & Prézelin, B. B. Spectral modeling of UV inhibition of *in situ* Antarctic primary production using a field derived biological weighting function. *Photochem. Photobiol.* **64**, 407–418 (1996).
13. Veth, C. The evolution of the upper water layer in the marginal ice zone, austral spring 1988, Scotia-Weddell Sea. *J. Mar. Syst.* **2**, 451–464 (1991).
14. Nelson, D. M. & Smith, W. O. Jr Sverdrup revisited: Critical depths, maximum chlorophyll levels, and the control of Southern Ocean productivity by the irradiance-mixing regime. *Limnol. Oceanogr.* **36**, 1650–1661 (1991).
15. Cullen, J. J. & Lesser, M. P. Inhibition of photosynthesis by ultraviolet radiation as a function of dose and dosage rate: Results for a marine diatom. *Mar. Biol.* **111**, 183–190 (1991).
16. Franks, P. J. S. & Marra, J. A simple new formulation for phytoplankton photoresponse and an application in a wind-driven mixed-layer model. *Mar. Ecol. Prog. Ser.* **111**, 145–153 (1994).
17. Yamazaki, H. & Kamykowski, D. The vertical trajectories of motile phytoplankton in a wind-mixed water column. *Deep-Sea Res.* **38**, 219–241 (1991).
18. Anis, A. & Moun, J. N. Surface wave-turbulence interactions: scaling $\epsilon(z)$ near the sea surface. *J. Phys. Oceanogr.* **25**, 2025–2045 (1995).
19. Prézelin, B. B., Boucher, N. P. & Smith, R. C. in *Ultraviolet Radiation in Antarctica: Measurements and Biological Effects* (eds Weiler, C. S. & Penhale, P. A.) 159–186 (Am. Geophys. Union, Washington DC, 1994).
20. Helbling, E. W., Villafañe, V., Ferrario, M. & Holm-Hansen, O. Impact of natural ultraviolet radiation on rates of photosynthesis and on specific marine phytoplankton species. *Mar. Ecol. Prog. Ser.* **80**, 89–100 (1992).
21. Zepp, R. G. & Cline, D. M. Rates of direct photolysis in aquatic environment. *Environ. Sci. Technol.* **11**, 359–366 (1977).
22. Murray, A. G. & Jackson, G. A. Viral dynamics II: a model of the interaction of ultraviolet light and mixing processes on virus survival in seawater. *Mar. Ecol. Prog. Ser.* **102**, 105–114 (1993).
23. Vincent, W. F. & Roy, S. Solar ultraviolet-B radiation and aquatic primary production: damage, protection and recovery. *Environ. Rev.* **1**, 1–12 (1993).
24. Morel, A. Available, usable, and stored radiant energy in relation to marine photosynthesis. *Deep-Sea Res.* **25**, 673–688 (1978).
25. Cullen, J. J. & Neale, P. J. in *The Effects of Ozone Depletion on Aquatic Ecosystems* (ed. Häder, D.-P.) 97–118 (Landes, Austin, 1997).
26. Gregg, W. W. & Carder, K. L. A simple spectral solar irradiance model for cloudless maritime atmospheres. *Limnol. Oceanogr.* **35**, 1657–1675 (1990).
27. Davis, R. F., Lazin, G., Bartlett, J., Ciotti, A. & Staben, P. Remote sensing of a pigment patch in the southeastern Bering Sea. *Proc. SPIE* **2963**, 654–657 (1997).
28. Prieur, L. & Sathyendranath, S. An optical classification of coastal and oceanic waters based on the specific absorption curves of phytoplankton pigments, dissolved organic matter, and other particulate materials. *Limnol. Oceanogr.* **26**, 671–689 (1981).
29. Sakshaug, E., Johnsen, G., Andersen, K. & Vernet, M. Modeling of light-dependent algal photosynthesis and growth: experiments with Barents Sea diatoms *Thalassiosira nordenskiöldii* and *Chaetoceros furcellatus*. *Deep-Sea Res.* **38**, 415–430 (1991).
30. Denman, K. L. & Gargett, A. E. Time and space scales of vertical mixing and advection of phytoplankton in the upper ocean. *Limnol. Oceanogr.* **28**, 801–815 (1983).

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Increased polar stratospheric ozone losses and delayed eventual recovery owing to increasing greenhouse-gas concentrations

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The chemical reactions responsible for stratospheric ozone depletion are extremely sensitive to temperature¹. Greenhouse gases warm the Earth's surface but cool the stratosphere radiatively^{2–5} and therefore affect ozone depletion. Here we investigate the interplay between projected future emissions of greenhouse gases and levels of ozone-depleting halogen species using a global climate model that incorporates simplified ozone-depletion chemistry. Temperature and wind changes induced by the

increasing greenhouse-gas concentrations alter planetary-wave propagation in our model, reducing the frequency of sudden stratospheric warmings in the Northern Hemisphere⁴. This results in a more stable Arctic polar vortex, with significantly colder temperatures in the lower stratosphere and concomitantly increased ozone depletion. Increased concentrations of greenhouse gases might therefore be at least partly responsible for the very large Arctic ozone losses observed in recent winters^{6–9}. Arctic losses reach a maximum in the decade 2010 to 2019 in our model, roughly a decade after the maximum in stratospheric chlorine abundance. The mean losses are about the same as those over the Antarctic during the early 1990s, with geographically localized losses of up to two-thirds of the Arctic ozone column in the worst years. The severity and the duration of the Antarctic ozone hole are also predicted to increase because of greenhouse-gas-induced stratospheric cooling over the coming decades.

Parametrized chemistry was included interactively in the Goddard Institute for Space Studies (GISS) Global Climate/Middle Atmosphere Model (GCMAM)¹⁰, a primitive equation model including parametrized gravity waves. This model has $8^\circ \times 10^\circ$ resolution (latitude $\times$ longitude), with 23 vertical layers extending from the surface to 85 km. Mountain wave drag was reduced globally to one-quarter its usual value¹¹ to allow realistic simulation of Southern Hemisphere polar lower-stratospheric temperatures, which were otherwise too warm. Modelled ozone transport was fixed at climatological values. This includes only zonal mean motions, so that we may underestimate the amount of chemical processing by longitudinally asymmetric motion of air across low-temperature regions. Ozone transport changes induced by increasing greenhouse gases were calculated non-interactively. We performed a control run without greenhouse gas forcing, and a run with forcings (Fig. 1), each including the chlorine loading trend^{1,12,13}. Both began in 1959, allowing time for removal of any influence of initial conditions, and continued to the end of 2070. The atmosphere was coupled to a mixed-layer ocean with specified

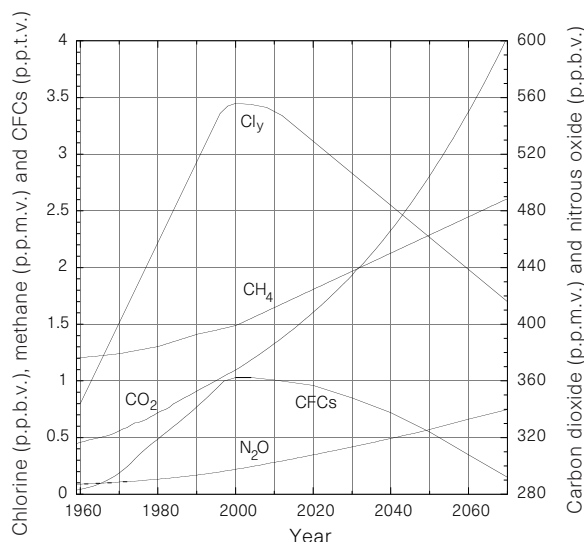


Figure 1 Emission trends for CO₂, N₂O, CH₄, CFCs and chlorine (Cly) used as input to the model runs. Projected lower stratospheric chlorine loading (independent of height or latitude) is based on the trend derived from the 1992 Copenhagen revisions to the 1987 Montreal Protocol on Substances that Deplete the Ozone Layer¹, normalized slightly upwards and decreasing more slowly in the future to match recent observations^{12,13,30}. Greenhouse-gas emissions are based on observations to the end of 1984, and subsequently are similar to, though for carbon dioxide slightly lower than, the 1992 International Panel on Climate Change (IPCC) preferred scenario IS92a²⁵. The exception is CFCs, for which a steady reduction after 2000 has been assumed, consistent with their expected phase-out and with the chlorine loading scenario.

ocean heat transports and diffusion through the base of the mixed layer, allowing sea surface temperatures to respond to greenhouse forcings⁴.

A simple, but physically realistic parametrization of the heterogeneous chemistry responsible for polar ozone depletion was used^{11,14}. Computational resources prohibit the use of more complex chemistry. Briefly, chlorine activation takes place whenever temperatures fall below 195 K, a rough threshold for formation of polar stratospheric cloud or supercooled sulphate aerosol, as observations and detailed photochemical calculations show rapid activation below this temperature regardless of phase and composition details of heterogeneous surfaces^{15,16}. Ozone depletion takes place at each location with active chlorine and sunlight according to photochemistry and temperature¹⁷. An additional contribution of 15% from bromine chemistry is also included. This parametrization reproduces quite well observed global chlorine monoxide

abundances¹⁸ and polar ozone losses¹¹, and has been used in previous studies of ozone response to climate change¹⁴. Other models using crude heterogeneous chemistry parametrizations have also obtained reasonable results for global-scale ozone loss^{19–21}. We note that should massive denitrification occur in the Arctic, ozone loss could be extended even beyond that modelled here.

Radiative cooling by increasing greenhouse gases by itself causes area-weighted temperature decreases of $\sim 1\text{--}2\text{ K}$ poleward of 70° from altitudes of 200 to 50 mbar during 2010–19 in the winter in both hemispheres, relative to the control run. In the Northern Hemisphere, the reduced frequency of stratospheric warmings adds to the radiative cooling, resulting in total temperature decreases within the enhanced Arctic vortex of $5\text{--}7\text{ K}$ during December and January. Large ozone losses in February and March exert a sizeable positive feedback, so that modelled temperatures are $8\text{--}10\text{ K}$ colder in the greenhouse run owing to combined radiative, dynamical and chemical influences.

In the Southern Hemisphere, the radiative cooling and the resulting positive feedback from increased chemical ozone loss lead to more persistent extremely cold temperatures. Although an Antarctic ozone hole occurs in the control run, November and December are 4 K colder during 2010–19 in the run with increasing greenhouse gases, prolonging the duration of the ozone hole. (The 1990 Antarctic ozone hole itself induced lower-stratospheric cooling of $\sim 6\text{ K}$ in October, and $\sim 8\text{ K}$ in November in the model¹¹, in good agreement with observations.) Total modelled cooling relative to pre-ozone-hole conditions in the 2010s is $\sim 7\text{ K}$ in October, and $\sim 12\text{ K}$ in November, similar to the Arctic values.

Figure 2 shows that Arctic ozone losses are greatest in the 2010s, while during the late 1990s and early 2000s they are similar in percentage to Antarctic losses observed in the late 1980s. Without climate forcing, average ozone destruction would only change within the model as a result of changes in chlorine amounts. Chlorine abundance in the 2010s is similar to 1990s levels, so that the much greater mean Arctic ozone loss in the 2010s displays the distinct signature of stratospheric cooling induced by increasing greenhouse gases. Greenhouse-gas-induced lower-stratospheric cooling also causes the Antarctic ozone hole to expand vertically and horizontally relative to its current state, as seen in equilibrium doubled CO_2 experiments^{11,19}. As ozone depletion is already very large, additional destruction over that seen in the current decade is fairly small. Recovery of ozone to early 1980s levels does not take place until roughly 2050.

Antarctic ozone depletion is up to $50\text{--}60\%$ of the column during 2010–19, and roughly symmetric around the South Pole (Fig. 3). Though interannual variability remains large, over 50% of the ozone column is also frequently destroyed over a large area of the Arctic. The total amount of ozone destroyed in the Arctic during this decade is $\sim 85\%$ of Antarctic destruction during the same decade. Over Greenland and Northern Europe however, more ozone is destroyed than anywhere within the Antarctic ozone hole.

In Fig. 4 we compare observed and modelled Antarctic and Arctic ozone column minima. Within the large variability, the model reproduces well the decreasing values seen in the 1990s in both hemispheres. The most severe Arctic depletions are seen during 2006–21, when column minima drop below 150 Dobson units (DU) during four of the coldest, most stable years.

Ozone transport differences between the control and experimental runs were calculated non-interactively¹⁴. The stronger Arctic polar vortex slows meridional transport across the vortex boundary, leading to a slight ozone build-up outside the vortex, with associated slight ozone decreases within the vortex. Additionally, increased residual circulation brings down more ozone into the highest altitudes where ozone depletion occurs. These effects change ozone amounts by less than 8% at any point during 2010–19; a small value relative to chemical losses. Southern Hemisphere

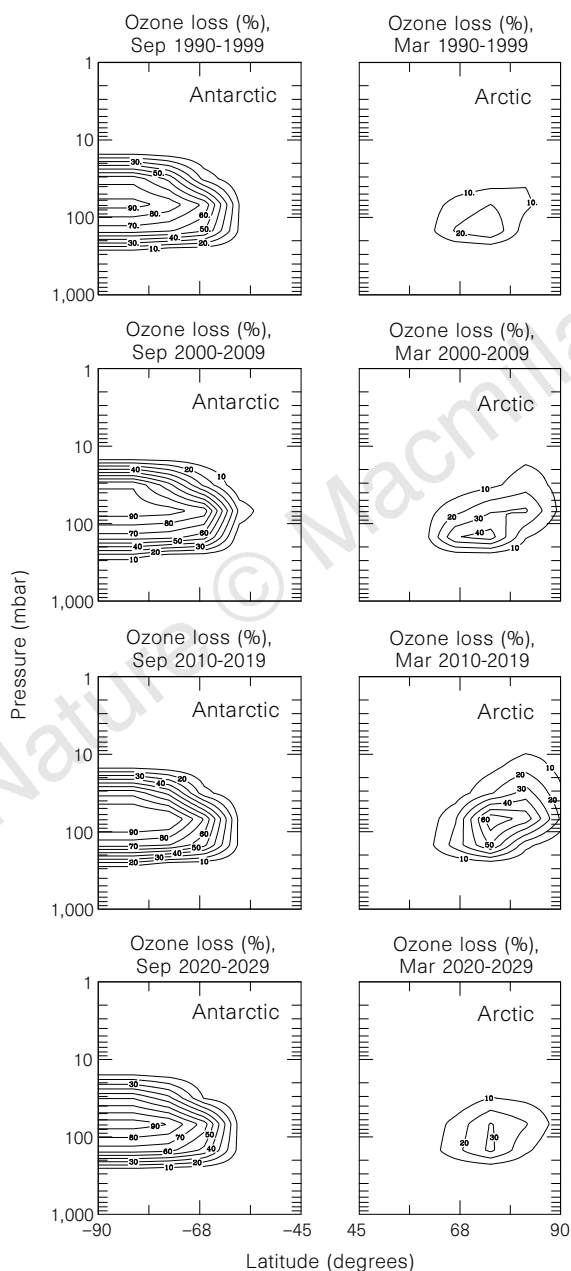


Figure 2 Decadally averaged vertical profiles of zonal mean ozone losses during September for the Southern Hemisphere and during March for the Northern Hemisphere. Values are percentage depletion with respect to pre-1980 observed climatological ozone.

changes are even smaller. In contrast, altered transports in an equilibrium doubled- CO_2 experiment with the GISS model changed Arctic ozone amounts by more than 25% (ref. 14), so the importance of transport changes is likely to increase significantly towards the middle of the next century.

Whereas climate models are fairly consistent in generating lower-stratospheric radiative cooling in response to increasing greenhouse gases, changes in planetary-wave energy propagation may be much more model-dependent. These experiments show that Arctic ozone loss is extremely sensitive to a reduction in the frequency of sudden stratospheric warmings, agreeing with earlier work demonstrating that the presence, or lack of, mid-winter sudden stratospheric warming is the crucial factor in determining whether an Arctic ozone hole will form under doubled- CO_2 conditions^{22,23}.

The GISS model generates a large tropical upper-tropospheric warming in response to increasing greenhouse gases: 10 K in the doubled- CO_2 experiments, 3 K during the 2010s in these experiments. This warming increases the latitudinal temperature gradient over the altitude range ~ 600 –100 mbar, causing the lower-stratospheric (300–10 mbar) zonal winds at 40 – 50°N during December–February to speed up by 9 – 11 m s^{-1} for doubled CO_2 , and 3 – 5 m s^{-1} during the 2010s in these experiments, relative to the control runs. Faster winds alter the refraction of planetary waves, limiting the ability of wave number two (planetary wave n is the wave with n nodes) to propagate up into the stratosphere⁴. Sudden warmings in our model are typically triggered by a combination of

waves one and two, especially major warmings (as has often been observed²⁴). The net result is a reduction in the frequency of stratospheric warmings despite an enhancement of wave one. This mechanism therefore depends upon the degree of tropical upper-tropospheric warming due to increased CO_2 generated by a particular model. We note that another GISS model with 31 vertical layers and $4^\circ \times 5^\circ$ resolution produces a similar upper-tropospheric warming, as does the 23-layer model with standard mountain drag, suggesting that this response is not very sensitive to model resolution or mountain drag. Whereas models such as the UK Meteorological Office model generate upper-tropospheric warmings similar to those of the GISS model, others (such as the models of the Geophysical Fluid Dynamics Laboratory or the National Center for Atmospheric Research) do not²⁵. The Météo-France group performed an experiment using prescribed temperature changes in response to CO_2 increases as calculated in the Hamburg model, which gave a weaker temperature response than is typical²⁵. Though they did not calculate polar ozone loss, they found an increase in the frequency of sudden stratospheric warmings with a doubling of atmospheric carbon dioxide⁵.

In support of the GISS model, observations generally agree with the trends seen in our simulations. The winter-time Arctic vortex area with temperatures below 195 K has increased considerably over the past three decades²⁶, as have winter-time lower-stratospheric zonal winds²⁷. Recent Arctic winters have followed the trends in our model, showing large ozone losses and an increasingly stable and

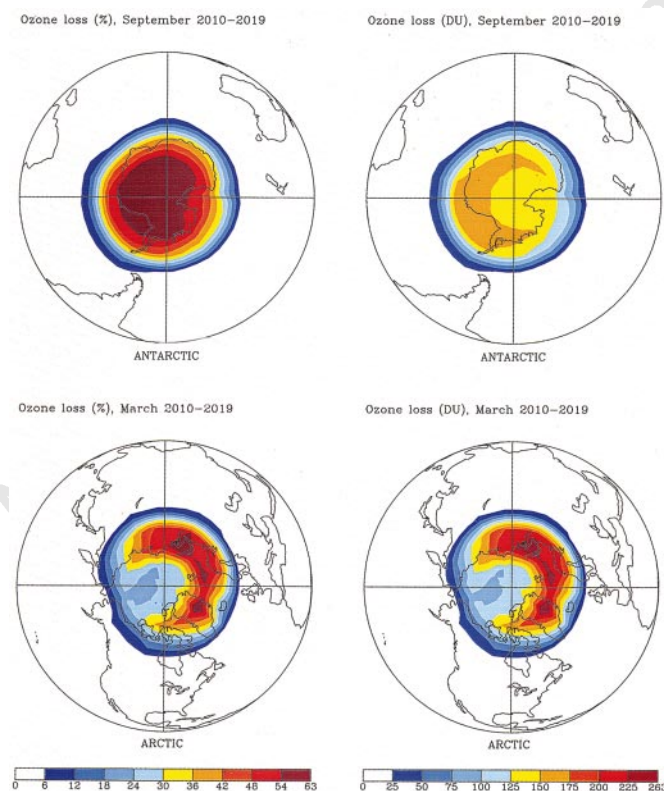


Figure 3 Total column ozone loss averaged over 2010–19, during September for the Southern Hemisphere (top) and March for the Northern Hemisphere (bottom). The left-hand panels show the total ozone column loss in per cent. In the Arctic, despite the enhanced polar vortex, decadal mean loss is very non-symmetric. The GISS GCMAM produces an Aleutian high-pressure region in good agreement with observations, resulting in colder temperatures on average from 90°W through 0° to 90°E . Average ozone losses are therefore greater in that region. As there is more springtime ozone in the Northern Hemisphere than in the Southern Hemisphere, in addition to showing the relative percentage losses, total column loss is also shown in Dobson units (DU; right-hand panels), indicating the absolute number of ozone molecules destroyed.

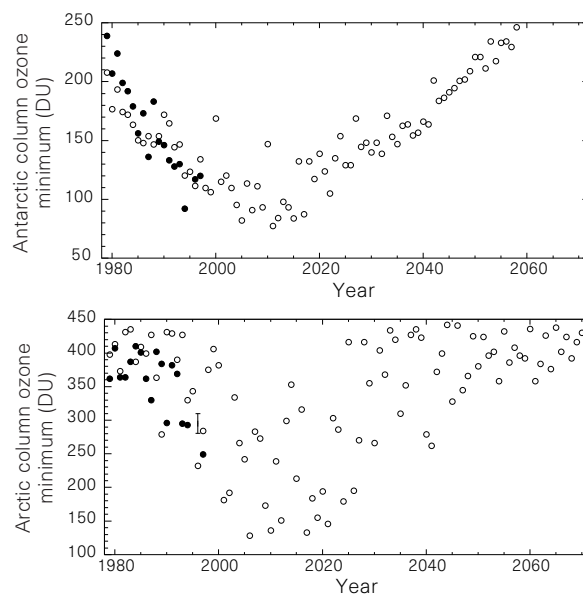


Figure 4 Total column ozone minima in the polar regions during spring. The top panel shows minimum ozone (DU) south of 65° averaged over the last three days of September (except for 1993, for which 23–25 September was used), as seen in TOMS version 7 data (filled circles), and in the model (open circles). Similarly, the bottom panel shows minimum column ozone north of 65° , averaged over the last three days of March, as seen in TOMS data and in the model. Model values do not include intrusions of low-ozone air from lower latitudes into the Arctic, so they tend to be on the high side of observed values. The bar shown for 1996 is an estimate for that year derived from chemical column depletions of 120–160 DU with respect to a climatological mean of 435 DU, calculated based on Halogen Occultation Experiment observations⁷.

persistent polar vortex^{6–9,28}. In the first eight years of the 1990s, there has been only one Arctic major warming during December–February, as compared with five during the 1980s.

The model results are triggered by changes in the tropopause-region latitudinal temperature gradient. This gradient can be increased by both greenhouse warming and by high-latitude ozone loss (recent trends^{26–28} have probably been influenced by both). High-latitude ozone loss provides a positive feedback loop, creating nonlinear dynamical and ozone responses to climate forcing. Observations of the temporal evolution of the tropopause-region latitudinal temperature gradient are therefore crucial. Combined with measurements of the frequency of sudden stratospheric warmings, they may verify our modelled chemistry–climate linkage which creates a severe Arctic ozone hole.

Despite worldwide limits on chlorofluorocarbon (CFC) production implemented after the discovery of the ozone hole over Antarctica²⁹, uncertainties remain about the exact timing of ozone-hole recovery. Our results suggest that increasing greenhouse gases may worsen the situation, leading to severe ozone depletion over the Arctic, as well as increasing the severity and duration of the Antarctic ozone hole. Furthermore, due to the influence of climate change, the maximum chemical depletion of polar ozone may occur roughly a decade after the maximum chlorine abundance in the stratosphere, and roughly 15 years after the decline in the emission of CFCs (recently observed in the troposphere for some compounds³⁰). Thus recovery of the Earth's ozone layer may take place later than currently expected. □

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1. Scientific Assessment of Ozone Depletion: 1994 (Rep. 37, World Meteorological Organization, Geneva, 1995).
2. Fels, S. B., Mahlman, J. D., Schwarzkopf, M. D. & Sinclair, R. W. Stratospheric sensitivity to perturbations in ozone and carbon dioxide: radiative and dynamical response. *J. Atmos. Sci.* **37**, 2265–2297 (1980).
3. Rind, D., Suozzo, R., Balachandran, N. K. & Prather, M. J. Climate change and the middle atmosphere. Part I: the doubled CO₂ climate. *J. Atmos. Sci.* **47**, 475–494 (1990).
4. Rind, D., Shindell, D. T., Loneragan, P. & Balachandran, N. K. Climate change and the middle atmosphere. Part III: the doubled CO₂ climate revisited. *J. Clim.* (in the press).
5. Mahfouf, J. F., Cariolle, D., Royer, J.-F., Geleyn, J.-E. & Timbal, B. Response of the Météo-France climate model to changes in CO₂ and sea surface temperature. *Clim. Dyn.* **9**, 345–362 (1994).
6. Manney, G. L., Santee, M. L., Froidevaux, L., Waters, J. W. & Zurek, R. W. Polar vortex conditions during the 1995–96 Arctic winter: Meteorology and MLS ozone. *Geophys. Res. Lett.* **23**, 3203–3206 (1996).
7. Müller, R. *et al.* Severe chemical ozone loss during the Arctic winter of 1995–96. *Nature* **389**, 709–712 (1997).
8. Rex, M. *et al.* Prolonged stratospheric ozone loss in the 1995–96 Arctic winter. *Nature* **389**, 835–838 (1997).
9. Newman, P. A., Gleason, J. F., McPeters, R. D. & Stolarski, R. S. Anomalous low ozone over the Arctic. *Geophys. Res. Lett.* **24**, 2689–2692 (1997).
10. Rind, D. *et al.* The GISS global climate/middle atmosphere model. Part I: model structure and climatology. *J. Atmos. Sci.* **45**, 329–370 (1988).
11. Shindell, D. T., Wong, S. & Rind, D. Interannual variability of the Antarctic ozone hole in a GCM. Part I: the influence of tropospheric wave variability. *J. Atmos. Sci.* **54**, 2308–2319 (1997).
12. von Clarmann, T. *et al.* Determination of the stratospheric chlorine budget in the spring arctic vortex from MIPAS B limb emission spectra and air sampling experiments. *J. Geophys. Res.* **100**, 13979–13997 (1995).
13. Woodbridge, E. L. *et al.* Estimates of total organic and inorganic chlorine in the lower stratosphere from in situ and flask measurements during AASE II. *J. Geophys. Res.* **100**, 3057–3064 (1995).
14. Shindell, D. T., Rind, D. & Loneragan, P. Climate change and the middle atmosphere. Part IV: ozone response to doubled CO₂. *J. Clim.* (in the press).
15. Shindell, D. T. & de Zafra, R. L. Limits on heterogeneous processing in the Antarctic spring vortex from a comparison of measured and modelled chlorine. *J. Geophys. Res.* **102**, 1441–1449 (1997).
16. Jaeglé, L. *et al.* Evolution and stoichiometry of heterogeneous processing in the Antarctic stratosphere. *J. Geophys. Res.* **102**, 13235–13253 (1997).
17. Shindell, D. T. & de Zafra, R. L. Chlorine monoxide in the Antarctic spring vortex 2. A comparison of measured and modelled diurnal cycling over McMurdo Station, 1993. *J. Geophys. Res.* **101**, 1475–1487 (1996).
18. Santee, M. L., Manney, G. L., Read, W. G., Froidevaux, L. & Walters, J. W. Polar vortex conditions during the 1995–96 Arctic winter: MLS ClO and HNO₃. *Geophys. Res. Lett.* **23**, 3207–3210 (1996).
19. Pitari, G., Palmeri, S., Visconti, G. & Prinn, R. G. Ozone response to a CO₂ doubling: results from a stratospheric circulation model with heterogeneous chemistry. *J. Geophys. Res.* **97**, 5953–5962 (1992).
20. Cariolle, D., Lasserre-Bogorzy, A., Royer, J. F. & Geleyn, J. F. A general circulation model simulation of the springtime Antarctic ozone decrease and its impact on mid-latitudes. *J. Geophys. Res.* **95**, 1883–1898 (1990).
21. Mahlman, J. D., Pinto, J. P. & Umscheid, L. J. Transport, radiative, and dynamical effects of the Antarctic ozone hole: A GFDL “SKYHI” model experiment. *J. Atmos. Sci.* **51**, 489–508 (1994).
22. Austin, J., Butchart, N. & Shine, K. Possibility of an Arctic ozone hole in a doubled-CO₂ climate. *Nature* **360**, 221–225 (1992).
23. Austin, J. & Butchart, N. The influence of climate change and the timing of stratospheric warmings on Arctic ozone depletion. *J. Geophys. Res.* **99**, 1127–1145 (1994).
24. O'Neill, A. & Pope, V. D. Simulation of linear and non-linear disturbances in the stratosphere. *Q. J. R. Meteorol. Sci.* **114**, 1063–1075 (1988).

25. Houghton, J. T., Callander, B. A. & Varney, S. K. (eds) *Climate Change 1992; The Supplementary Report to the IPCC Scientific Assessment* (Cambridge Univ. Press, 1992).
26. Pawson, S. & Naujokat, B. Trends in daily wintertime temperatures in the northern stratosphere. *Geophys. Res. Lett.* **24**, 575–578 (1997).
27. Kodera, K. & Koide, H. Spatial and seasonal characteristics of recent decadal trends in the northern hemispheric troposphere and stratosphere. *J. Geophys. Res.* **102**, 19433–19447 (1997).
28. Zurek, R. W., Manney, G. L., Miller, A. J., Gelman, M. E. & Nagatani, R. M. Interannual variability of the north polar vortex in the lower stratosphere during the UARS mission. *Geophys. Res. Lett.* **23**, 289–292 (1996).
29. Farman, J. C., Gardiner, B. G. & Shanklin, J. D. Large losses of total ozone in Antarctica reveal seasonal ClO_x/NO_x interaction. *Nature* **315**, 207–210 (1985).
30. Montzka, S. A. *et al.* Decline in the tropospheric abundance of halogen from halocarbons: Implications for stratospheric ozone depletion. *Science* **272**, 1318–1322 (1996).

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Variability of the path of the Kuroshio ocean current over the past 25,000 years

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The Kuroshio current is the strong northwestern component of the subtropical North Pacific Ocean gyre, and advects a large amount of heat from the tropics to northern mid-latitudes. The Kuroshio has bimodal stationary flow patterns, with small and large meander paths east of central Japan^{1,2} which switch on annual and decadal timescales^{3,4}. These switches seem to be caused by changes in current velocity and volume transport of the North Equatorial Current that are associated with variations in the trade-wind intensity in the eastern equatorial North Pacific Ocean^{5,6}. Here we present alkenone-derived sea surface temperature records at multicentennial resolution from sediment cores from the Nishishichitou ridge off central Japan. These 25,000-year records show that the Kuroshio path has also fluctuated on millennial timescales. This variability resembles that of the subtropical high pressure of the North Pacific, reconstructed from terrestrial pollen distributions, water levels in North American lakes, and marine micropalaeontological records⁷. Together, these data indicate that climate variability off central Japan over the past 25,000 years may be part of a circum-Pacific phenomenon, reflecting the rate of subtropical surface circulation in the North Pacific Ocean.

Piston cores were collected from three locations (KT92-17 stations 14, 19 and 20) on the Nishishichitou ridge off central Japan, to reconstruct climate change in the northwestern North Pacific (Fig. 1). The surface hydrographic conditions around the ridge are controlled largely by latitudinal migration of the current axis of the Kuroshio, known as its small and large meander paths (Fig. 1). With the small meander path, a warm water mass derived from the Kuroshio occupies the surface layer around station 14. Thus, water temperatures around this station have often been significantly higher than those to the south, creating a counter-current of the Kuroshio with a geostrophic balance around stations 19 and 20 (ref. 8). With the large meander path, a large cold surface water mass is established off central Japan which lowers sea surface temperatures (SST) around station 14. These two flow patterns are both stable, and alternate over short timescales^{3,4}.

The unsaturation ratio of C₃₇ alkenones (U₃₇^{k'}; see definition⁹ in

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Fig. 2 legend) was investigated as a proxy for temperature change in this oceanic region over the past 25 kyr. The sediment cores were sectioned into 2.4-cm-thick slices for alkenone analysis¹⁰. Haptophytes, the only organisms known to synthesize long-chain alkenones¹¹, grow in both surface and subsurface waters through

the seasonal thermocline (~100 m depth)¹². However, U_{37}^k -based temperatures in this oceanic region are held to be more representative of SST, as elsewhere¹³, because of a significant correlation between measured SSTs and U_{37}^k -based temperatures of sediment trap samples from the northwestern North Pacific off central Japan¹⁴. Ages were based on accelerator mass spectrometry (AMS)-¹⁴C determinations of planktonic foraminifera *Neogloboquadrina dutertrei*, corrected for reservoir age¹⁵.

Sea surface temperatures (based on U_{37}^k measurements) in the core samples from stations 14, 19 and 20 from 25 to 10 kyr before present (BP) were lower than present values and tended to increase through the Holocene (Fig. 2b–d). This trend was similar to the variations of temperature calculated from the transfer function

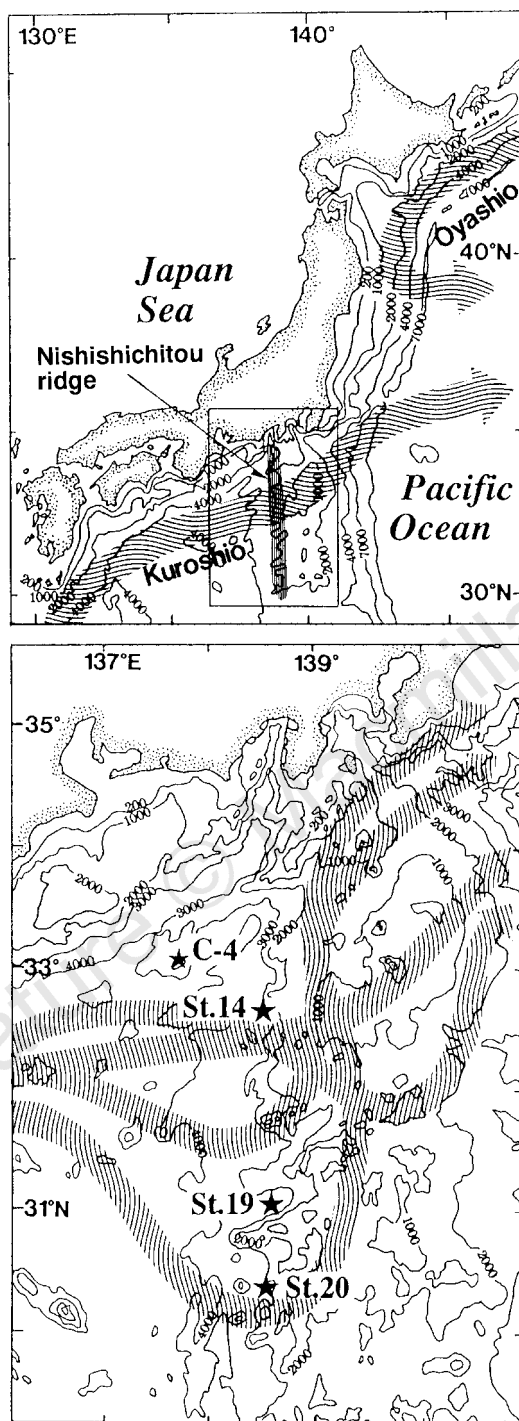


Figure 1 Sampling locations of sediment cores used in this work. KT92-17 station 14 (32° 40.1' N, 138° 27.3' E, water depth 3,252 m), station 19 (31° 5.7' N, 138° 39.9' E, water depth 3,336 m) and station 20 (30° 22.6' N, 138° 38.9' E, water depth 3,280 m) were from the Nishishichitou ridge in the northwestern North Pacific off central Japan. Diagonal hatched lines represent the general paths of the Kuroshio current in the direction of the arrows, based on Nitani¹. Surface sediment samples (0–1 cm depth) were also collected by a multiple corer at the same site as station 20. The sampling location of sediment core C-4 (33° 9.0' N, 137° 41.9' E, water depth 3,343 m)¹⁶ is also shown.

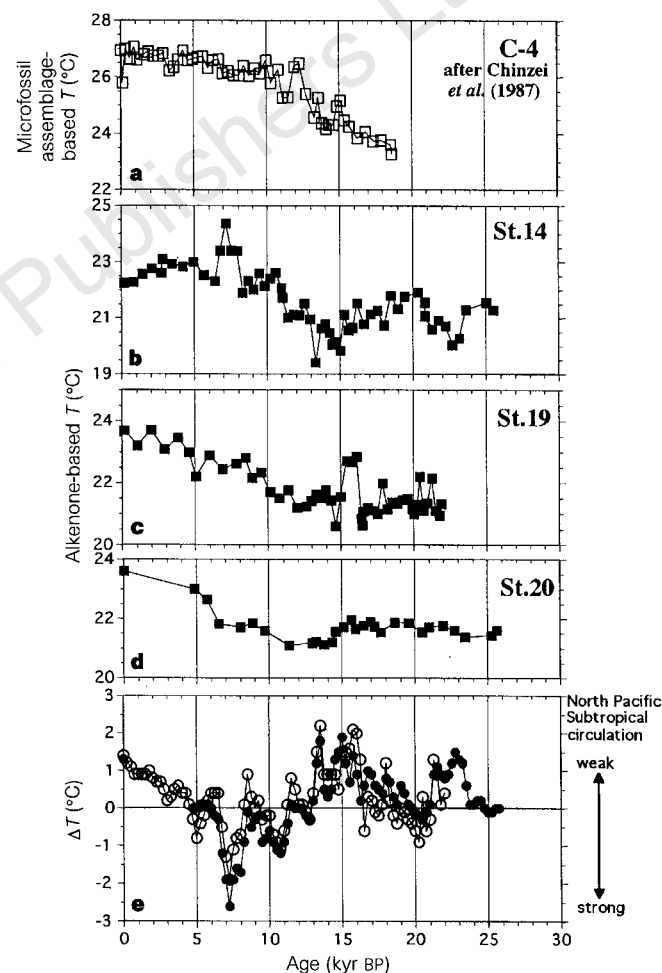


Figure 2 Past temperatures from core data. **a**, variations in temperature (T ; □) based on microfossil assemblages of nannoplankton, diatoms, planktonic foraminifera and radiolaria in core C-4¹⁶; **b–e**, Variations in U_{37}^k -based SST ($^{\circ}\text{C}$; ■) in cores from station 14 (**b**), station 19 (**c**) and station 20 (**d**) on the Nishishichitou ridge. Ages of all sediment samples were calculated by linear interpolation, based on AMS ¹⁴C ages using a 350 years reservoir correction¹⁵. The unsaturation ratio of C_{37} alkenones (U_{37}^k) was calculated as $U_{37}^k = [C_{37:2}]/([C_{37:2}] + [C_{37:3}])$ (ref. 9), where $[C_{x:y}]$ denotes the concentration of the long-chain alkenone with x carbon atoms and y double bonds. Temperatures were calculated by using the following equation: $U_{37}^k = 0.034T + 0.039$ (ref. 9). The validity of this U_{37}^k -temperature relationship for the northwestern North Pacific sediments was confirmed by analyses of alkenone in particulate organic matter¹⁴. The precision of the measurements ($\pm 2\sigma$) was generally better than 0.02 U_{37}^k unit (or 0.5 $^{\circ}\text{C}$) based on duplicates of the same sediment samples. **e**, South–north latitudinal variations in temperature difference (ΔT) calculated by subtracting the interpolated (250 years) SST value on the basis of U_{37}^k -based temperature for the station 14 core from the corresponding values of station 20 (●) or station 19 (○).

based on the microfossil assemblage in an adjacent core (core C-4: Figs 1 and 2a)¹⁶. U_{37}^k -based SSTs at station 14 were, however, about 2–4.5 °C lower than the summer temperatures at C-4 based on microfossil assemblage (Fig. 2a and 2b). This discrepancy may reflect the inherent bias of U_{37}^k -based temperature values to record SST during maximal alkenone production of the spring bloom, as observed in the sediment-trap investigations off central Japan¹⁴. The temperature differences between 25–13 kyr BP and the present estimated by U_{37}^k in station 14 (1.5–2.5 °C) was smaller than the estimates based on microfossil assemblages at C-4 (4.0 °C). Colder ocean temperatures observed at C-4 from 25 to 13 kyr BP are attributed to the southward shift of the cold water mass associated with the confluence zone of the Kuroshio and Oyashio waters¹⁶. However, the microfossil assemblage in the C-4 core included planktonic foraminifera which inhabit the intermediate layer (~400 m depth)¹⁷, and therefore are not strictly comparable to alkenone records. Furthermore, salinity analysis of the water mass in this area¹⁸ indicates that the cold water of the Oyashio often intrudes below the euphotic zone when it extends south of 35° N. It is unlikely, therefore, that U_{37}^k -based SST would reflect the southward position of this cold water mass between 25 and 13 kyr BP. The difference in values of U_{37}^k -based temperature between 25–13 kyr BP and the present (1.5–2.5 °C) are comparable to those reported for mid-latitude North Pacific waters¹⁹. Thus, such lower temperatures may reflect the same phenomenon as global cooling in the Northern Hemisphere²⁰.

Distinct excursions in U_{37}^k -based temperature were seen at station 14 (during 14–13 and 8–6.5 kyr BP) and station 19 (during 16.5–15.5 kyr BP) (Fig. 2b–d), but none was observed in the microfossil record in C-4 core. These data therefore reflect sea surface environmental changes, which may have been caused by migration of the Kuroshio. Temporal variations in the Kuroshio off central Japan were examined using south to north latitudinal differences in SST (ΔT) along the Nishishichitou ridge calculated by subtracting the U_{37}^k -based temperature of the station 14 core from temperatures derived from stations 19 and 20, respectively (Fig. 2e). ΔT for both core-top samples was 1.3 °C, comparable to the modern SST difference²¹, but has fluctuated over the past 25 kyr. The episodes of negative ΔT from 26 to 24.5 and 21 to 19 kyr BP suggest that the axis of the Kuroshio current moved northwards in association with a small amount of meandering off central Japan. Maximum ΔT

occurs from 16 to 15 and from 13.5 to 13 kyr BP, indicating a southward deflection of the Kuroshio associated with large meandering, although the values are <1 °C higher than at present. ΔT decreases from ~13 kyr BP to reach a pronounced minimum at 7 kyr BP. When the SST at station 14 was almost 2.7 °C higher than at both station 20 and 19, this was taken as convincing evidence that the Kuroshio had moved northwards. The reduced meander path during this period corresponds with the middle Holocene. After 7 kyr BP, ΔT tends to increase to its present level.

The current velocity and volume transport of the Kuroshio are critical factors in determining its flow pattern off central Japan. They are closely related to the intensity of the North Equatorial Current, which, in turn, is influenced by trade-wind strength in the eastern equatorial North Pacific^{5,6}. A barotropic inflow–outflow model⁶ suggests that low velocities for the Kuroshio (Rossby number $Ro < 1.75$) lead to large meanders, whereas intermediate Rossby values ($1.75 < Ro < 1.93$) favour variable meandering with rapid interchange of the two paths. Higher Rossby numbers ($Ro > 1.93$) lead to small meanders. Strong trade winds can therefore lead to intensification of North Equatorial Current, and consequently high Ro for the Kuroshio, which results in small meanders.

Millennial-scale fluctuations in the flow pattern of the Kuroshio were possibly provoked by variation in the intensity of trade winds associated with the subtropical North Pacific high-pressure, which is controlled by albedo and specific heat balance in the North American continent. COHMAP members⁷ demonstrated that the subtropical high pressure was displaced to the south and weakened from about 18 to 12 kyr BP because of the widespread Laurentide ice sheet over North America. It then intensified gradually during melting and reduction of the Laurentide ice sheet due to increased solar radiation in summer during deglaciation. Boreal summer insolation reached a maximum during the middle Holocene, when subtropical high pressure must also have peaked⁷. Thus, intensification of the subtropical high pressure may have strengthened subtropical North Pacific circulation, which, in turn, led to northward migration of the Kuroshio. These changes may therefore be linked at a millennial scale. The pronounced minimum ΔT from 8 to 7 kyr BP suggests that the subtropical North Pacific circulation may have reached a maximum. We assume that during the ΔT minimum, called 'Kuroshio culmination', the Kuroshio advected heat from the tropics to the mid-latitude Pacific most efficiently. Fluctuations due to El Niño and non-El Niño conditions in the equatorial eastern North Pacific could also act as a trigger for the change in the flow pattern of the Kuroshio⁵. However, El Niño events are annual to decadal phenomena, and they cannot account for millennial-scale fluctuations in the flow pattern of the Kuroshio.

Our determinations of latitudinal SST gradients off central Japan can provide a history of the subtropical North Pacific surface circulation. Until now, records of grain size of aeolian dust in pelagic sediments²², palaeoproductivity^{23,24} and SST²⁵ in upwelling regions have been used as indicators of wind intensity. However, it is difficult to reconstruct millennial-scale fluctuations from dust records because of the low sedimentation rates of pelagic sediments. Also, upwelling proxies such as palaeoproductivity and SST appear to be better suited for reconstruction of the intensities of local currents rather than those of global-scale geostrophic currents like the Kuroshio. Accumulation-rate records of organic carbon and biogenic silica used as palaeoproductivity proxies are particularly affected by biological control and efficiency of transport and preservation²⁶. We suggest that U_{37}^k measurements may in the future provide a better means of assessing variations in latitudinal heat transport by western boundary currents. □

Table 1 ^{14}C ages of the three sediment cores

Station no.	^{14}C datings/Aira-Tanzawa volcanic ash		
	Depth from core top (cm)	Conventional age (yr BP)	Corrected age§ (yr BP)
14	24.8–27.3	3,810 ± 116	3,430
	58.4–60.9	6,745 ± 209	6,365
	150.6–153.1	14,055 ± 143	13,675
	203.4–205.9	16,509 ± 264	16,129
	285–299.5*	24,330 ± 225*	23,950
19	86.4–88.9	8,172 ± 205	7,822
	142.2–144.7	12,996 ± 262	12,646
	310.1–312.6	20,246 ± 241	19,896
	421.0–423.5	22,232 ± 844†	21,882
20	0.0–2.5	5,218 ± 299‡	4,868
	44.4–46.9	13,183 ± 194	12,833
	122.6–125.1	7,563 ± 697‡	17,213
	170.6–173.1	22,516 ± 315	22,166
	194.6–211.3*	24,088 ± 1209‡	23,738†

Sediments of the Nishishichitou ridge consisted of homogeneous olive-greenish silty clay with several volcanic ash and turbidite layers (station 14: 35–46.4, 163–175, 285–300 cm depth; station 19: 36–62.4, 225.7–237.7 cm depth; station 20: 194.6–211.2 cm depth)²⁷.

* Aira-Tanzawa (AT) ash layers: the age has been determined previously as 24,330 ± 225 yr BP (ref. 28).

† ^{14}C ages measured in this study were used for the AT ash layer in station 20.

‡ ^{14}C ages were determined from the planktonic foraminifera *N. eggeri* and *Globigerina bulloides* in addition to *N. dutertrei*.

§ Correction of reservoir effect assumed as 350 years in the area from 30° to 33° N of the North Pacific¹⁵.

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1. Nitani, H. Variation of the Kuroshio south of Japan. *J. Oceanogr. Soc. Jpn* **31**, 154–173 (1975).
2. Kawabe, M. Sea level variations at the Izu Islands and typical stable paths of the Kuroshio. *J. Oceanogr. Soc. Jpn* **41**, 307–326 (1985).

3. Kawabe, M. Spectral properties of sea level and time scales of Kuroshio path variations. *J. Oceanogr. Soc. Jpn* **43**, 111–123 (1987).
4. Kimura, S. & Sugimoto, T. Short-period fluctuations in meander of the Kuroshio's path off Cape Shiono Misaki. *J. Geophys. Res.* **98**, 2407–2418 (1993).
5. Yamagata, T., Shibao, Y. & Umatani, S. Interannual variability of the Kuroshio Extension and its relation to the Southern Oscillation/El Niño. *J. Oceanogr. Soc. Jpn* **41**, 274–281 (1985).
6. Yasuda, I., Yoon, J. H. & Sugimoto, T. Dynamics of the Kuroshio large meander-Barotropic model. *J. Oceanogr. Soc. Jpn* **41**, 259–273 (1985).
7. COHMAP Members. Climatic changes of the last 18,000 years: observations and model simulations. *Science* **241**, 1043–1052 (1988).
8. Kaneko, A. H., Mizuno, S., Koterayama, W. & Gordon, R. L. Cross-stream velocity structures and their downstream variation of the Kuroshio around Japan. *Deep-Sea Res.* **39**, 1583–1594 (1992).
9. Prahl, F. G., Muehlhausen, L. A. & Zahnle, D. L. Further evaluation of long-chain alkenones as indicators of paleoceanographic conditions. *Geochim. Cosmochim. Acta* **52**, 2303–2310 (1988).
10. Sawada, K., Handa, N., Shiraiwa, Y., Danbara, A. & Montani, S. Long-chain alkenones and alkyl alkenoates in the coastal and pelagic sediments of the northwest North Pacific with special reference to the reconstruction of *Emiliania huxleyi* and *Gephyrocapsa oceanica* ratios. *Org. Geochem.* **24**, 751–764 (1996).
11. Marlowe, I. T., Brassell, S. C., Eglinton, G. & Green, J. C. Long-chain alkenones and alkyl alkenoates and the fossil coccolith record of marine sediments. *Chem. Geol.* **88**, 349–375 (1990).
12. Balch, W. M., Holligan, P. M., Ackleson, S. G. & Voss, K. J. Biological and optical properties of mesoscale coccolithophore blooms in the Gulf of Maine. *Limnol. Oceanogr.* **36**, 629–643 (1991).
13. Sikes, E. L. & Keigwin, L. D. Equatorial Atlantic sea surface temperature for the last 30 kyr: A comparison of U_{37}^* , $\delta^{18}O$ and foraminiferal assemblage temperature estimates. *Paleoceanography* **9**, 31–45 (1994).
14. Sawada, K., Handa, N. & Nakatsuka, T. Production and transport of long-chain alkenones and alkyl alkenoates in sea water column in the northwestern North Pacific off central Japan. *Mar. Chem.* (in the press).
15. Bard, E. Correction of accelerator mass spectrometry ^{14}C ages measured in planktonic foraminifera: paleoceanographic implications. *Paleoceanography* **3**, 635–645 (1988).
16. Chintzei, K. et al. Postglacial environmental change of the Pacific Ocean off the coasts of central Japan. *Mar. Micropaleontol.* **11**, 273–291 (1987).
17. Fairbanks, R. G. & Wiebe, P. H. Foraminifera and chlorophyll maximum: vertical distribution, seasonal succession and paleoceanographic significance. *Science* **209**, 1524–1526 (1980).
18. Yang, S.-K., Nagata, Y., Taira, K. & Kawabe, M. Southward intrusion of the Intermediate Oyashio Water along the east coast of the Boso Peninsula, Japan II. Intrusion events into Sagami Bay. *J. Oceanogr. Soc. Jpn* **49**, 173–191 (1993).
19. CLIMAP Project Members. The surface of the ice-age earth. *Science* **191**, 1131–1137 (1976).
20. Broecker, W. S. & Denton, G. H. The role of ocean-atmosphere reorganizations in glacial cycles. *Geochim. Cosmochim. Acta* **53**, 2465–2501 (1989).
21. Japan Oceanographic Data Center. *Marine Environmental Atlas, Northwestern Pacific Ocean II* (Japan Hydrographic Assoc., Tokyo, 1978).
22. Rea, D. K. The paleoclimatic record provided by eolian deposition in the deep sea: the geologic history of wind. *Rev. Geophys.* **32**, 159–195 (1994).
23. Rea, D. K., Pisias, N. G. & Newberry, T. Late Pleistocene paleoclimatology of the central equatorial Pacific: Flux patterns of biogenic sediments. *Paleoceanography* **6**, 227–244 (1991).
24. Farrell, J. W., Pedersen, T. F., Calvert, S. E. & Nielsen, B. Glacial-interglacial changes in nutrient utilization in the equatorial Pacific Ocean. *Nature* **377**, 514–517 (1995).
25. Lyle, M. W., Prahl, F. G. & Sparrow, M. A. Upwelling and productivity changes inferred from a temperature record in the central equatorial Pacific. *Nature* **355**, 812–815 (1992).
26. Berger, W. H. & Herguera, J. C. in *Primary Productivity and Biogeochemical Cycle in the Sea* (eds Falkowski, P. G. & Woodhead, A. D.) 455–485 (Plenum, New York, 1992).
27. Murayama, M., Ahagon, N., Hyong, S., Kanamatsu, T. & Taira, A. Lithology in sediment cores collected during the cruises of KT92-17 and KT93-17 (IGBP). *Kaiyou Monthly* **26**, 434–439 (1994). (in Japanese)
28. Murayama, M. et al. Re-examination of the eruption age of Aira-Tn Ash (AT) obtained from a piston core off Shikoku—determined by AMS ^{14}C dating of planktonic foraminifera. *J. Geol. Soc. Jpn* **99**, 787–798 (1993). (in Japanese with English abstract).

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Dissociation of the neural correlates of implicit and explicit memory

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One presentation of a word to a subject is enough to change the way in which the word is processed subsequently, even when there is no conscious (explicit) memory of the original presentation. This phenomenon is known as implicit memory^{1–3}. The neural correlates of implicit memory have been studied previously^{4–11}, but they have never been compared with the correlates of explicit

memory while holding task conditions constant or while using a procedure that ensured that the neural correlates were not 'contaminated' by explicit memory. Here we use scalp-recorded event-related brain potentials to identify neural activity associated with implicit and explicit memory during the performance of a recognition memory task. Relative to new words, recently studied words produced activity in three neuroanatomically and functionally dissociable neural populations. One of these populations was activated whether or not the word was consciously recognized, and its activity therefore represents a neural correlate of implicit memory. Thus, when task and memory contamination effects are eliminated, the neural correlates of explicit and implicit memory differ qualitatively.

Memory encoding was manipulated by cueing subjects to perform either a 'shallow' or a 'deep' study task. Depth of processing affects the ability of a subject to recollect a study episode consciously, but has little influence on measures of 'data-driven' implicit memory, such as repetition priming². In the first two experiments, studied (old) and unstudied (new) words were presented in a recognition memory test, and the event-related brain potentials (ERPs) produced by the different classes of test word were recorded. Crucially, we compared the ERPs produced by new words with those produced by old words that were misclassified by the subjects as new, reasoning that differences between these two classes of ERP would reflect memory in the absence of awareness.

Very similar results were obtained in each experiment and we report the data collapsed across the two studies ($n = 30$). Of the

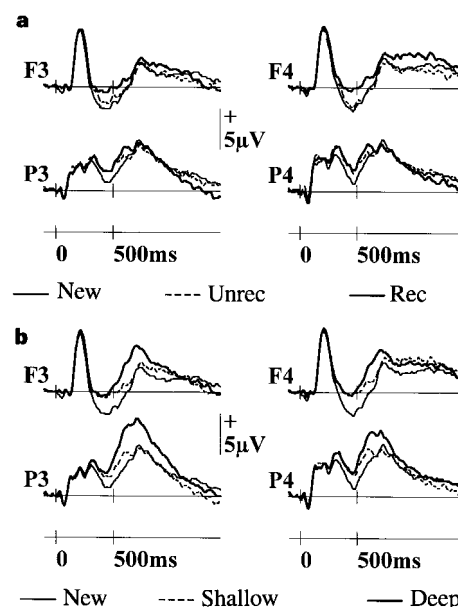


Figure 1 ERP waveforms, averaged across subjects, from left and right frontal (F3, F4 of the 10–20 system²⁶) and parietal (P3, P4) electrodes. **a**, ERPs elicited by correctly classified new words, and by unrecognized (unrec) and recognized (rec) shallowly studied words. ANOVA (factors of frontal versus parietal location, hemisphere and word type) of mean amplitudes between 300 and 500 ms revealed a location $\times$ word-type interaction is a standard statistical term, no explanation needed ($F_{2,53} = 5.97$, $P < 0.005$). Contrasts between unrecognized and new words restricted to the parietal electrodes revealed effects that were reliable for each experiment (1 and 2) separately ($F_{1,14} = 5.16$ and 6.52, both P values < 0.005) as well as for the combined data set ($F_{1,28} = 11.45$, $P < 0.005$). **b**, ERPs elicited by correctly classified new words, and by recognized shallowly and deeply studied words. ANOVA of 300–500-ms amplitudes revealed an effect of word type ($F_{2,56} = 18.19$, $P < 0.001$). There was no such effect for analysis of the data from the two types of old word. ANOVA of the 500–800-ms region revealed a significant word type $\times$ hemisphere interaction ($F_{2,46} = 10.82$, $P < 0.001$). This interaction remained when the ANOVA was restricted to data from the two types of old word ($F_{1,28} = 28.84$, $P < 0.001$).

deeply studied words, 94% were recognized, as compared with 49% of the shallowly studied items ($t_{29} = 26.27$, $P < 0.001$). 86% of the new words were correctly classified as new.

ERPs produced by shallowly studied words are shown in Fig. 1a, and are separated according to the accuracy of recognition judgement. The scalp topographies of the respective memory effects (differences between ERPs produced in response to studied and new words) are illustrated in Fig. 2a, b. From roughly 300 to 500 ms after the onset of the stimulus, ERPs from frontal electrode sites were more positive for recognized items than they were either for new words or for old words misclassified as new. During the same latency range (300–500 ms post-stimulus), ERPs from parietal electrodes showed a different pattern: regardless of the accuracy of the recognition judgement, old words produced more positive-going waveforms than did new words. This effect, which was equivalent in size for recognized and unrecognized items, is a neural correlate of memory in the absence of conscious recognition. As shown in Fig. 3, the effect was insensitive not only to accuracy of recognition judgement, but also to depth of processing. These functional properties identify the effect as a correlate of implicit memory.

In Fig. 1b we contrast the ERPs produced by new items with those elicited by recognized words. Between approximately 300 and 500 ms after the onset of the stimulus, memory effects for the two classes of studied word were equivalent in magnitude (Fig. 3) and scalp topography (Fig. 2a, c). From ~500 ms onwards, the ERPs to deeply studied words differed from those to both new and shallowly studied items. The scalp topography of this effect differed reliably from the memory effect elicited by the same words in the earlier, 300–500 ms, latency region (Fig. 2c, d), indicating that the two effects reflect the activity of distinct neural populations.

Our findings show that old words in a recognition memory test can produce three different patterns of memory-related activity. The functional characteristics of two of the patterns correspond closely to the characteristics of two kinds of memory—implicit

memory and conscious recollection—that have been distinguished on the basis of behavioural and neuropsychological evidence^{2,3}. Indeed, the ERP correlate of recollection identified here closely resembles that identified in previous studies^{11–14}. The third pattern of activity, maximal over the frontal scalp from 300–500 ms after the onset of the stimulus, is less easily characterized. This was found only for recognized old items, but was insensitive to depth of study processing. It may reflect item ‘familiarity’, a form of explicit memory held to be dissociable from recollection^{15–19}.

Our results go beyond previous findings by showing directly that neural activity elicited by recently experienced words that are not consciously recognized differs from activity elicited by genuinely new words. An important question is whether this effect merely represents weak explicit memory that is sufficient to be manifest in ERPs but too weak to lead to a positive recognition judgement^{14,20}. This possibility can be rejected on two grounds. First, the magnitude of the proposed neural correlate of implicit memory did not vary between recognized and unrecognized words, whereas a correlate of explicit memory should vary with recognition accuracy. Second, unrecognized and recognized words produced qualitatively different patterns of neural activity, indicating that explicit memory may involve the engagement of neural populations that are separate from those supporting memory without awareness.

If the ERP differences produced in response to new and unrecognized words are a correlate of implicit memory, similar effects should be seen in the kind of task standardly used to study implicit memory². In two other experiments, we investigated the ERPs produced by old and new words in a semantic judgement task, where it was irrelevant whether the words were old or new. A reliable repetition priming effect was observed on reaction time (old words, 891 ms, new words, 907 ms; $F_{1,30} = 7.19$, $P < 0.02$). This effect did not vary with depth of study processing. A positive-going memory effect was seen in ERPs at ~300–600 ms after stimulus onset (Figs 3 and 4). In its scalp distribution, time

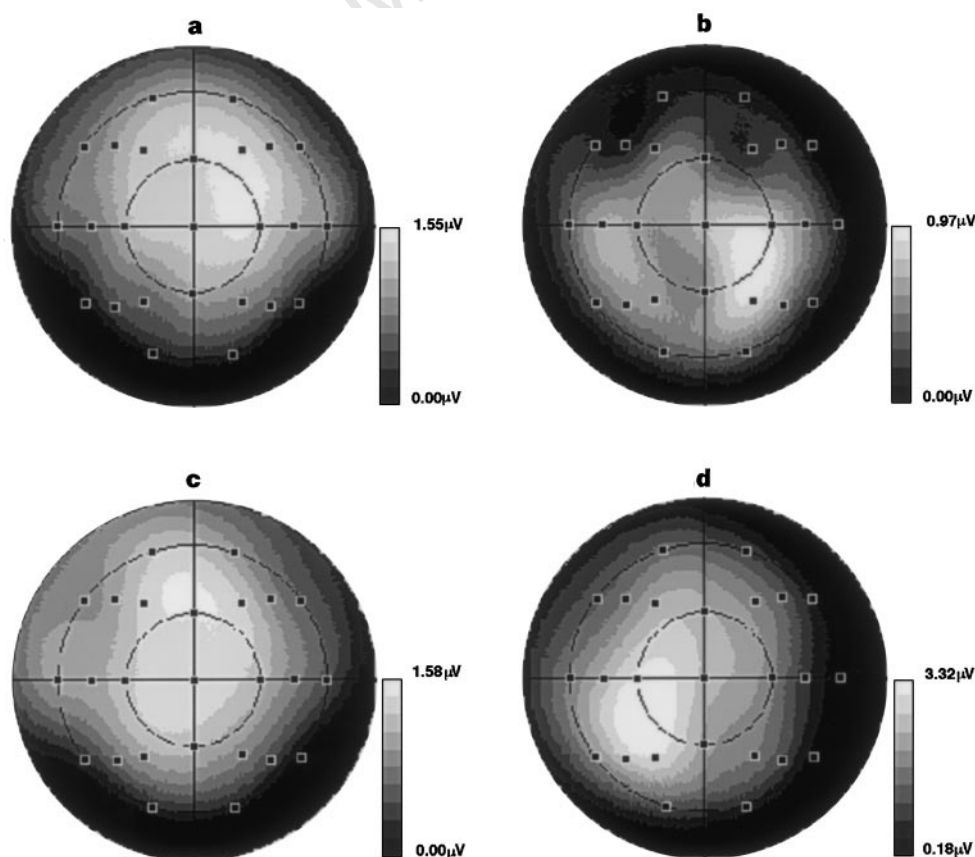


Figure 2 Scalp distributions²⁷ of the differences between the ERPs to new words and different classes of studied word. **a**, Recognized shallowly studied minus new, 300–500 ms post-stimulus. **b**, Unrecognized shallowly studied minus new, 300–500 ms post-stimulus. **c**, Recognized deeply studied minus new, 300–500 ms post-stimulus. **d**, Recognized deeply studied minus new, 500–800 ms post-stimulus. ANOVA revealed that the contrasts between **a** and **b**, and between **c** and **d**, were significant (**a** vs **b**: site $\times$ word type interaction $F_{4,115} = 4.11$, $P < 0.005$; **c** vs **d**: site $\times$ latency region interaction $F_{4,115} = 4.14$, $P < 0.005$). The contrast between **a** and **c** was not significant.

course, and insensitivity to depth of processing, the effect resembles that elicited by unrecognized old words in the first two experiments. Thus, the neural correlate of implicit memory identified in those experiments is also present when test items are not intentionally used as retrieval cues.

Our findings are the first demonstration that the neural correlates of implicit and explicit memory can be dissociated within a single task. They provide strong support for the view^{21,22} that these two forms of memory reflect the operation of qualitatively distinct neural systems. □

Methods

Recognition task. Subjects ($n = 16$ and 18 in experiments 1 and 2, respectively) were healthy, young, right-handed adults, naive to the purpose of the experiment. In each experiment, 15 subjects provided enough data (>16 artefact-free trials) to form ERPs for both correctly classified items and shallowly studied items misclassified as new.

Two (experiment 1) or three (experiment 2) study test blocks were used. In each study phase, 68 critical and 4 filler words were presented one at a time on a TV monitor. Each study word was preceded by a cue which indicated the nature of the task to be performed on that word. In response to one cue, subjects determined whether the first and last letters of the word were in alphabetic order (shallow task). In response to the other cue, they incorporated the word into a short sentence (deep task). After ~ 5 min, the test task was administered. The 68 critical words from the study phase were presented, randomly intermixed with 34 critical unstudied words and 10 unstudied fillers. The words (presentation duration 300 ms, maximum visual angle 1.5×0.4 degrees (experiment 1); or 500 ms, 2.8×0.5 degrees (experiment 2)) were presented every 5.3 s (experiment 1) or 5.5 s (experiment 2). Each word was preceded for 2.1 s by a fixation character, which was erased 102 ms before the word's onset. Subjects discriminated between old and new items as quickly and as accurately as possible by pressing one of two response keys. The study and test lists were constructed so that, across subjects, each critical item served equally often as a word that was new, deeply studied, and shallowly studied. The words ranged

Figure 3 Magnitudes of ERP memory effects. **a, b**, Mean ($\pm$ s.e.m.) differences in the amplitude of the 300–500 (**a**) and 500–800 ms (**b**) latency regions of ERPs to new words and ERPs to recognized deeply studied words and recognized and unrecognized shallowly studied words, shown for left (F3, P3) and right (F4, P4) frontal and parietal electrodes. **c**, Mean ($\pm$ s.e.m.) of the differences in the semantic judgement task between the amplitude of the 300–500-ms latency region of ERPs to new words and ERPs to deeply and shallowly studied old words. Double asterisks indicate that these results differ from zero with $P < 0.005$; single asterisks indicate that these results differ from zero with $P < 0.05$.

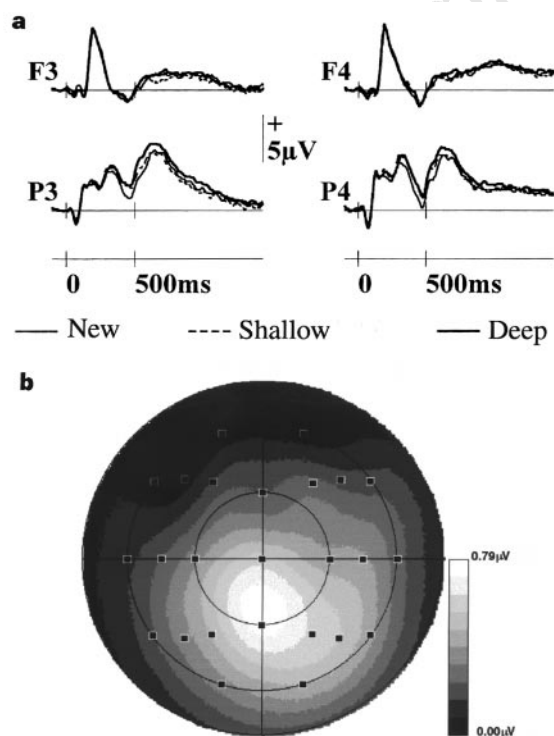
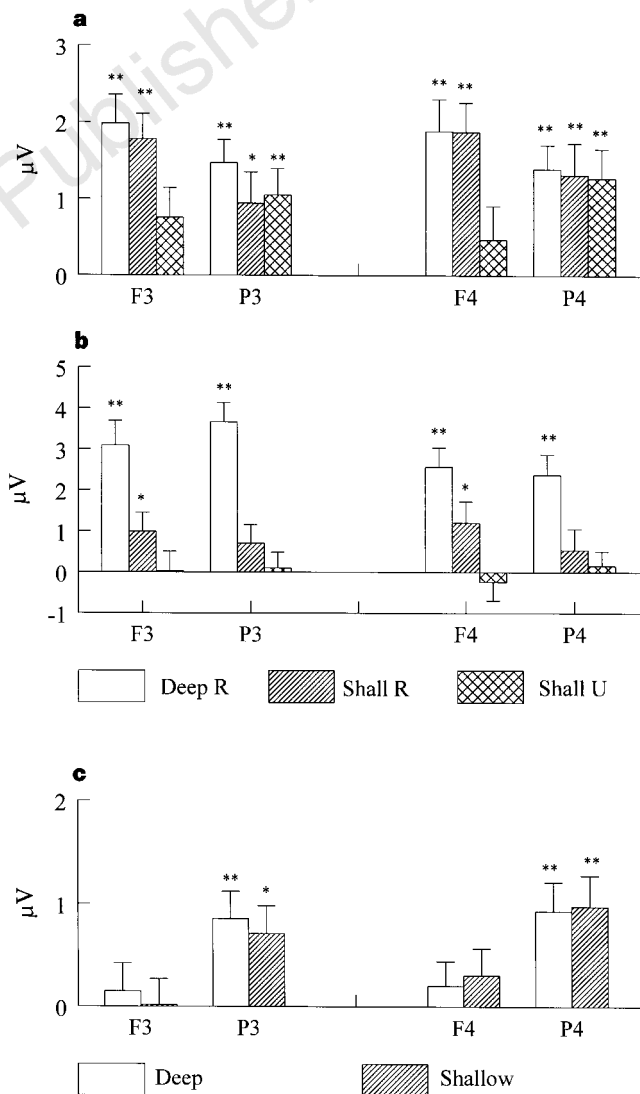


Figure 4 The semantic judgement task. **a**, ERPs from frontal (F3, F4) and parietal (P3, P4) electrodes in the semantic judgement task. ANOVA of the 300–500-ms latency region revealed a location $\times$ word-type interaction ($F_{2,58} = 5.51$, $P < 0.01$). Contrasts between word types restricted to the parietal electrodes revealed effects that were reliable for both experiments (3 and 4) separately ($F_{2,28} = 4.17$, $F_{2,24} = 3.59$, values $P < 0.05$) and for the combined data set ($F_{2,60} = 7.73$, $P < 0.001$). **b**, Scalp topography of the ERP differences between old (collapsed over study conditions) and new words.



from low to medium frequencies of occurrence, and from four to nine letters in length.

ERPs (sampling rate 6 ms per point, epoch length 1,536 ms, prestimulus baseline 102 ms) produced in response to different classes of test word were obtained from 25 scalp sites as described²³. ERPs were quantified by measuring the mean amplitude of two latency regions, 300–500 and 500–800 ms after the onset of stimulus. Amplitude differences were assessed by analysis of variance (ANOVA) (degrees of freedom (d.f.) corrected for non-sphericity²⁴). Differences in scalp topography were assessed by d.f.-corrected ANOVA of the data from all 25 electrodes after rescaling²⁵.

Semantic judgement task. Two groups of young adults ($n = 16$) were used in each experiment. For experiment 3, the subjects were the same individuals as those used in experiment 1. A new sample was used in experiment 4.

Experimental items were drawn from the same word pool used to construct the lists for experiments 1 and 2. For experiment 3, two study test blocks identical in structure to those used in experiment 1, although using different items, were administered. The only difference in procedure from experiment 1 was that subjects were required during the test to classify each test word as animate or inanimate. The procedure for experiment 4 was identical to that for experiment 3 except that the depth of processing manipulation at study was blocked. Stimulus display parameters and electroencephalogram recording for these experiments were as for experiment 1. As the differences between old and new words in the two experiments were very similar, and there were no block effects in experiment 4, the data are reported collapsed across experiments.

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- Graf, P. & Schacter, D. L. Implicit and explicit memory for new associations in normal and amnesic subjects. *J. Exp. Psychol. Learn. Mem. Cogn.* **11**, 501–518 (1985).
- Roediger, H. L. & McDermott, K. B. in *Handbook of Neuropsychology* Vol. 8 (eds Boller, F. & Grafman, J.) 63–131 (Elsevier, Amsterdam, 1993).
- Moscovitch, M., Vriezen, E. & Goshen-Gottstein, Y. in *Handbook of Neuropsychology* Vol. 8 (eds Boller, F. & Grafman, J.) 133–173 (Elsevier, Amsterdam, 1993).
- Squire, L. R. *et al.* Activation of the hippocampus in normal humans: a functional anatomical study of memory. *Proc. Natl Acad. Sci. USA* **89**, 1837–1841 (1992).
- Buckner, R. L. *et al.* Functional anatomical studies of explicit and implicit memory retrieval tasks. *J. Neurosci.* **15**, 12–29 (1995).
- Schacter, D. L., Savage, C. R., Alpert, N. M., Rauch, S. L. & Albert, M. S. Conscious recollection and the human hippocampal formation: evidence from positron emission tomography. *Proc. Natl Acad. Sci. USA* **93**, 321–325 (1996).
- Schacter, D. L. *et al.* Brain regions associated with the retrieval of structurally coherent visual information. *Nature* **376**, 587–590 (1995).
- Buckner, R. L. *et al.* Functional-anatomic correlates of object priming in humans revealed by rapid presentations fMRI. *Neuron* **20**, 285–296 (1998).
- Allan, K., Wilding, E. L. & Rugg, M. D. Electrophysiological evidence for dissociable processes contributing to recollection. *Acta Psychologica* **98**, 231–252 (1998).
- Paller, K. A. & Gross, M. Brain potentials associated with perceptual priming versus explicit remembering during the repetition of visual word-form. *Neuropsychologia* (in the press).
- Paller, K. A., Kutas, M. & McIsaac, H. K. An electrophysiological measure of priming of visual word-form. *Conscious. Cogn.* (in the press).
- Paller, K. A. & Kutas, M. Brain potentials during retrieval provide neurophysiological support for the distinction between conscious recollection and priming. *J. Cog. Neurosci.* **4**, 375–391 (1992).
- Smith, M. E. Neurophysiological manifestations of recollective experience during recognition memory judgements. *J. Cog. Neurosci.* **5**, 1–13 (1993).
- Rugg, M. D. in *Electrophysiology of Mind: Event-related Potentials and Cognition* (eds Rugg, M. D. & Coles, M. G. H.) 132–170 (Oxford Univ. Press, Oxford, 1995).
- Gardiner, J. M. & Java, R. I. in *Theories of Memory* (eds Collins, A. F., Gathercole, S. E., Conway, M. A. & Morris, P. E.) 163–188 (Earlbaum, Hove, 1993).
- Gardiner, J. M., Java, R. I. & Richardson-Klavehn, A. How level of processing really influences awareness in recognition memory. *Can. J. Exp. Psychol.* **50**, 114–122 (1996).
- Mandler, G. Recognizing: the judgment of previous occurrence. *Psychol. Rev.* **87**, 252–271 (1980).
- Yonelinas, A. P. Receiver operating characteristics in recognition memory: evidence for a dual-process model. *J. Exp. Psychol. Learn. Mem. Cogn.* **20**, 1341–1354 (1994).
- Hintzman, D. L. & Curran, T. Retrieval dynamics of recognition and frequency judgments: evidence for separate processes of familiarity and recall. *J. Mem. Lang.* **33**, 1–18 (1994).
- Ostergaard, A. L. & Jernigan, T. L. in *Implicit Memory: New Directions in Cognition, Development, and Neuropsychology* (eds Graf, P. & Masson, M. E. J.) 327–349 (Erlbaum, Hillsdale, NJ, 1993).
- Squire, L. R. *Memory and Brain* (Oxford Univ. Press, Oxford, 1987).
- Tulving, E. & Schacter, D. L. Priming and human memory systems. *Science* **247**, 301–306 (1990).
- Allan, K. & Rugg, M. D. An event-related potential study of explicit memory on tests of word-stem cued recall and recognition memory. *Neuropsychologia* **35**, 387–397 (1997).
- Winer, B. J. *Statistical Principles in Experimental Design* 2nd edn (McGraw-Hill, New York, 1971).
- McCarthy, G. & Wood, C. C. Scalp distributions of event-related potentials: an ambiguity associated with analysis of variance models. *Electroenceph. Clin. Neurophysiol.* **62**, 203–208 (1985).
- Jasper, H. A. The ten-twenty system of the international federation. *Electroenceph. Clin. Neurophysiol.* **10**, 371–375 (1958).
- Perrin, F., Pernier, J., Bertrand, O., Giard, M. H. & Echallier, J. F. Mapping of scalp potentials by surface spline interpolation. *Electroenceph. Clin. Neurophysiol.* **66**, 75–81 (1987).

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A cortical representation of the local visual environment

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Medial temporal brain regions such as the hippocampal formation and parahippocampal cortex have been generally implicated in navigation^{1–6} and visual memory^{7–9}. However, the specific function of each of these regions is not yet clear. Here we present evidence that a particular area within human parahippocampal cortex is involved in a critical component of navigation: perceiving the local visual environment. This region, which we name the 'parahippocampal place area' (PPA), responds selectively and automatically in functional magnetic resonance imaging (fMRI) to passively viewed scenes, but only weakly to single objects and not at all to faces. The critical factor for this activation appears to be the presence in the stimulus of information about the layout of local space. The response in the PPA to scenes with spatial layout but no discrete objects (empty rooms) is as strong as the response to complex meaningful scenes containing multiple objects (the same rooms furnished) and over twice as strong as the response to arrays of multiple objects without three-dimensional spatial context (the furniture from these rooms on a blank background). This response is reduced if the surfaces in the scene are rearranged so that they no longer define a coherent space. We propose that the PPA represents places by encoding the geometry of the local environment.

In the first experiment, nine right-handed students were scanned with fMRI while viewing 5.5-min videotapes in which scrambled and intact versions of black and white photographs of faces, common objects, houses and scenes were presented in separate epochs (Fig. 1a). Subjects either viewed the photographs passively, or performed a 'one-back' repetition detection task on the same stimuli (see Methods) which obliged them to attend to all stimuli irrespective of inherent interest. In all nine subjects, significantly greater activation was found during presentation of intact scenes than during presentation of intact faces and objects in the PPA, a bilateral region of parahippocampal cortex straddling the collateral sulcus (including the posterior tip of the parahippocampal gyrus and adjacent regions of the fusiform gyrus; Fig. 2). This region does not include the hippocampus proper.

For each subject individually, we used an independent data set from the same scanning session functionally to define a region of interest in the PPA (see Methods). We then extracted the time course of the per cent signal change relative to a fixation baseline within each subject's PPA over the period of the scan. To distinguish changes in activation resulting from high-level differences between the stimulus types from changes in activation resulting from low-level feature differences between the stimulus types, we subtracted the per cent signal change for the scrambled photographs from that for the intact photographs for each stimulus type before comparing the response across stimulus types. Analysis of variance found this activation difference between intact and scrambled photographs to be significantly greater for scenes than for houses ($F(1, 8) = 35.4$, $P < 0.001$), but not significantly greater for houses than for objects ($F(1, 8) = 3.4$, $P = 0.1$). Although the 1-back task was more difficult for scrambled than intact images, the pattern of the PPA response did not differ between the two tasks ($F < 1$), so the greater response to scenes is unlikely to be due to differences in attention or perceptual effort. These results demonstrate that the PPA responds selectively to visually presented scenes even when the response to some of the low-level features (such as local texture and average

luminance) of the stimuli has been subtracted out, and even when subjects merely view the stimuli passively with no task requirement.

The preference of the PPA for photographs of scenes over faces, objects and houses is suggestive, but does not in itself demonstrate that the PPA encodes spatial layout. If the PPA is involved in representing the shape of the local environment, then it should be strongly activated by scenes even when they depict only bare spatial layouts without discrete objects. On the other hand, if the PPA is involved in an analysis of the identities, meanings, or relative locations of the objects in the scenes, then it should be more active for arrays containing multiple objects (without any three-dimensional spatial context) than for bare spatial layouts. To assess these and other possibilities, six subjects were run in a second experiment in which they viewed (Fig. 3a) photographs of (1) unfamiliar indoor scenes of rooms with furniture, plants, and so on; (2) the same rooms photographed from the same angle after all of the objects had been removed; (3) arrays, each of which contained all of the objects from one of the furnished rooms cut out from the original background and rearranged in a random configuration; (4) faces; (5) single objects; (6) familiar outdoor scenes (of the MIT campus); (7) outdoor scenes of unfamiliar natural environments containing few discrete objects; and (8) familiar landmarks (mostly buildings) from the MIT campus cut out from their original backgrounds.

Strikingly, the PPA responded much more strongly to scenes depicting bare spatial layout (empty rooms and landscapes) than it

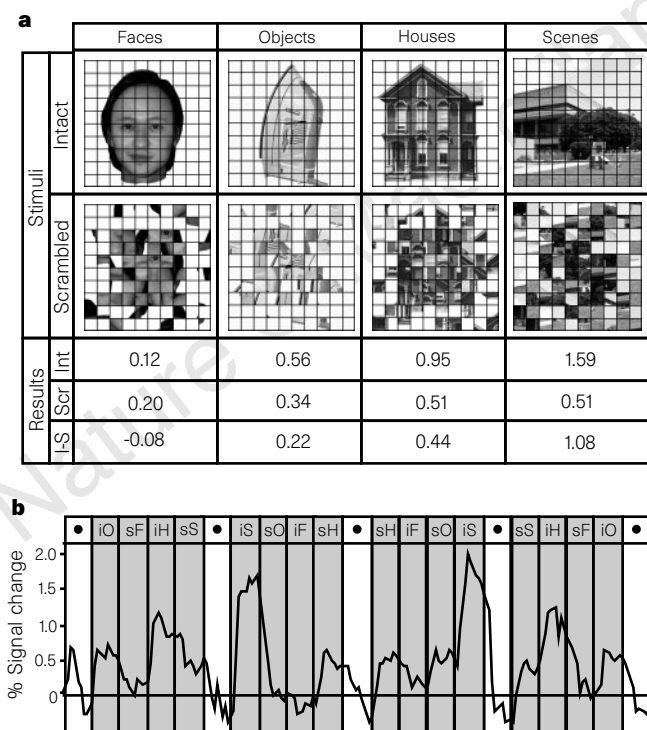


Figure 1 Results of experiment 1, demonstrating that the PPA responds selectively to scenes. **a**, Examples of intact and scrambled versions of the four different types of stimuli (top), and the average per cent signal change for each stimulus type in the PPA averaged over all subjects (bottom). The difference between intact and scrambled versions of each picture is a measure of the response in the PPA to each stimulus type partially unconfounded from the response to its low-level visual features. Half of the scenes were outdoor scenes of the MIT campus, and half were indoor scenes of unfamiliar locations. **b**, The time course of the per cent change in MR signal intensity in the PPA over the period of the scan. Per cent signal change was calculated individually for each subject using that subject's fixation activation as baseline and then averaging across subjects (black dot indicates fixation epochs). i, Intact; s, scrambled; S, scenes; F, faces; O, objects; H, houses.

did to faces, objects or multiple object arrays (Fig. 3). The response in the PPA to empty rooms was as strong as the response to the same rooms furnished ($F < 1.3$), and over twice as strong as the response to arrays of multiple objects without spatial context ($F(1,8) = 24.13$, $P < 0.01$). Further, the response to multiple object arrays was not significantly greater than to single objects ($F < 1$). Finally, the response to empty landscapes with few discrete objects was comparable to the response to empty rooms ($F < 1$). These results demonstrate that the presence of multiple objects is neither sufficient nor necessary for activation of the PPA. On the other hand, scenes depicting the shape of the local environment activate the PPA even if they are bare and uninteresting.

The response to landmarks cut out from their background was significantly higher than the response to objects ($F(1,8) = 28.0$, $P < 0.01$). The landmarks were mostly buildings, so this result is

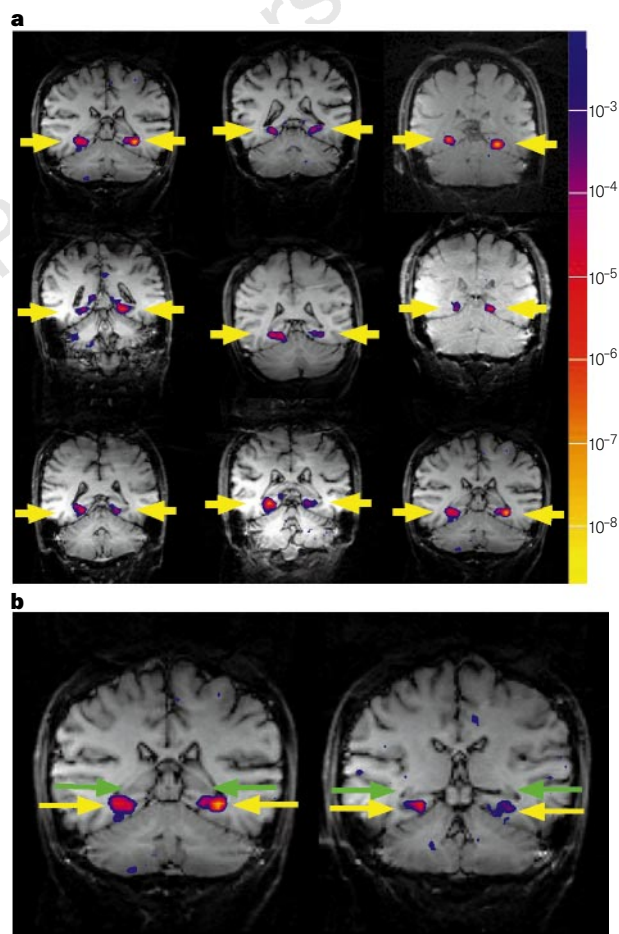


Figure 2 Anatomical location of the PPA. **a**, A single slice from each of the nine subjects in experiment 1 showing the PPA; functional data from this experiment is overlaid on a high-resolution T1-weighted anatomical image of the same slice. Right hemisphere appears on the left. Significance levels reflect the results of a Kolmogorov–Smirnov test comparing the MR signal intensity during viewing of intact scenes to signal intensity during viewing of intact objects and faces. Note that the location of the PPA (indicated with yellow arrows) is strikingly consistent across subjects. The activated region (contiguous voxels reaching the $P < 10^{-4}$ statistical criterion) was larger ($t(8) = 3.21$, $P < 0.01$) in the right hemisphere (average, 1.1 c.c.) than the left hemisphere (0.69 c.c.). Significant activation was also found in the anterior calcarine sulcus, but because of the proximity of this region to retinotopic cortex, this activation is not discussed here. **b**, Two adjacent slices from a single subject demonstrating that the PPA (yellow arrows) does not overlap with the posterior part of the hippocampus (green arrows). Posterior slice appears on the left. Talairach coordinates of PPA activation for this subject are -6 , 18 , -39 and -6 , -34 , 30 (S–I, M–L, A–P).

Figure 3 Results of experiment 2. **a**, Examples of the 8 different stimulus types with average per cent signal change in the PPA for each. **b**, Time course of the raw per cent change in MR signal averaged over all subjects. F, faces; O, objects; MO, multiple-object arrays; LM, landmarks; LS, landscapes; ER, empty rooms; FR, furnished rooms; OS, MIT outdoor scenes.

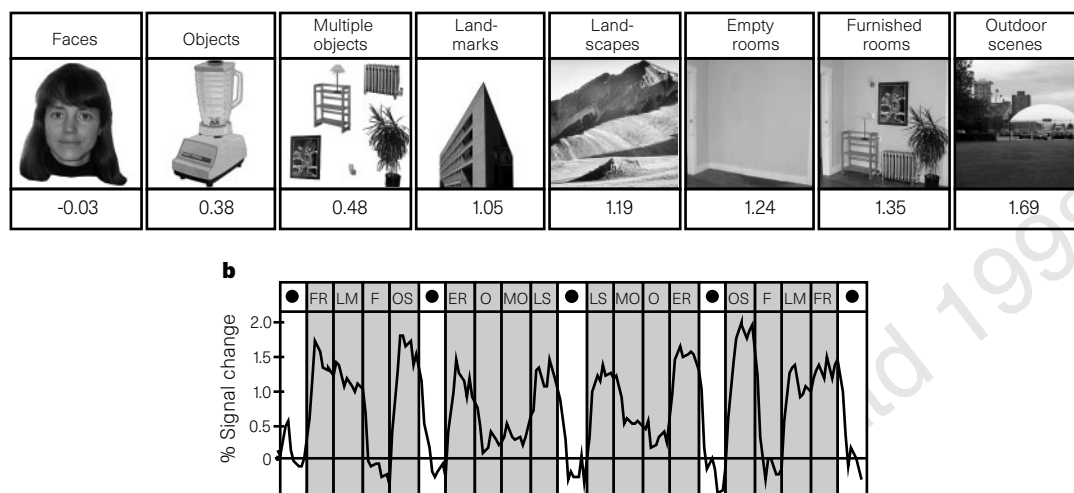
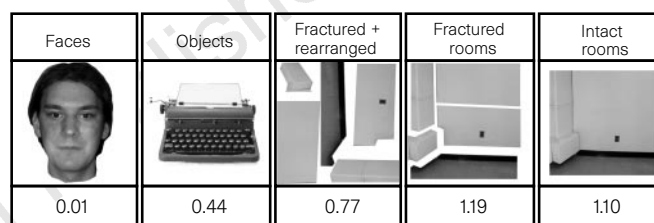


Figure 4 Results of experiment 3, showing the 5 different stimulus types with average per cent signal change in the PPA for each. (Subjects also viewed three other stimulus conditions designed to test other hypotheses; these are not reported here.)



consistent with previous work showing that buildings without background can evince a large response in cortical regions near the collateral sulcus^{10,11}. This result could reflect the fact that buildings are large stable structures that define the geometry of local space, or the fact that the subjects were familiar with the spatial context of the landmarks.

In a final test of the spatial-layout hypothesis, we investigated whether the response in the PPA would be reduced if the surfaces in the stimulus did not define a coherent space. Five subjects viewed images of a new set of empty rooms which were 'fractured' into their component surfaces (Fig. 4). In one stimulus condition (fractured rooms), the relative positions of the resulting surfaces were preserved. In another stimulus condition (fractured + rearranged), the relative positions of the resulting surfaces were rearranged so that they no longer defined a space. Subjects also viewed the original unfractured photographs of the rooms (intact rooms), single objects and faces.

The results showed a striking confirmation of the hypothesis: the PPA responded more strongly to both the intact ($F(1, 4) = 8.8$, $P < 0.05$) and the fractured rooms ($F(1, 4) = 19.5$, $P = 0.01$) than to the fractured + rearranged rooms, whereas the response to the fractured rooms was not significantly different from the response to the intact rooms ($F(1, 4) = 2.9$, $P > 0.15$). As both the fractured and the fractured + rearranged stimuli contained the same image components, the difference in response must reflect the fact that the fractured rooms defined a coherent space, whereas the fractured + rearranged rooms did not. Interestingly, even the jumbled surfaces in the fractured + rearranged rooms activated the PPA more than single objects ($F(1, 4) = 20.8$, $P = 0.01$); however, the response was clearly greater when these surfaces defined a space.

These experiments show that (1) there is a region of parahippocampal cortex that responds selectively to visual scenes depicting places; (2) activation in this region occurs automatically even when no explicit cognitive test is required; (3) this response is found even if there are no discrete objects in the scene; and (4) the critical factor in this response is the presence in the stimulus of information about the layout of local space. Our findings dovetail with two other lines

of research. First, behavioural studies have shown that disorientated human infants^{12,13} and rats^{14–16} reorientate themselves solely on the basis of the geometry of the local visual environment, ignoring non-spatial cues unless these have been proven by experience to be stable over time^{17,18}. Some of the results^{12–16} have been taken as evidence for a phylogenetically and developmentally primitive 'geometric module'¹⁹ which uses the shape of the surroundings to determine current location. Second, neuropsychological studies have shown that patients with damage to parahippocampal cortex often experience great difficulty finding their way around novel environments^{20–23}. Together with our results, these findings suggest that the PPA performs an analysis of the shape of the local environment that is critical to our ability to determine where we are. □

Methods

Subjects. Ten right-handed students from the Massachusetts Institute of Technology (MIT) performed both experiment 1 and experiment 2. One subject was omitted because of excessive head motion. Because the experimental protocol for experiment 2 was changed after the first three subjects were run, data from these subjects were not included in the analysis of experiment 2. Six right-handed students (from MIT or Harvard) performed experiment 3. One subject was omitted because of excessive head motion.

Materials. Stimuli were digitized black-and-white photographs. Scrambled pictures were created by dividing intact pictures into 100 square sections and then randomizing locations of squares within three concentric rings around fixation.

Procedure. The procedure for all experiments was as follows. Each subject was run on 2–3 scans for both the passive and 1-back tasks for each experiment. For the 1-back task, subjects were instructed to press a button whenever they saw two identical pictures in a row; this task was designed to ensure that subjects attended at least as much to uninteresting stimulus sets (for example, scrambled images) as to the more interesting sets (for example, faces and scenes). Each scan lasted 5 min and 36 s and consisted of sixteen 16-s epochs with fixation periods interleaved as shown in Figs 1b, 3b. During each epoch, 20 different photographs of the same type were shown (with 1 or 2 consecutive repetitions per epoch in the 1-back task). Each photograph was presented for 300 ms followed by a blank interval of 500 ms. There were two epochs for each of the eight stimulus types within each scan; order was counterbalanced over

two versions of each experiment (ABCD-EFGH-HGFE-DCBA for version 1 and HGFE-DCBA-ABCD-EFGH for version 2). The raw data from version 1 were rearranged so that the time courses from the two versions were compatible and could be averaged together.

MRI acquisition. Scanning was done on the 1.5 T (experiments 1 and 2) and 3 T (experiment 3) scanners at the MGH-NMR Center in Charlestown, Massachusetts, using a bite bar to minimize head motion and a bilateral surface coil which provided a high signal-to-noise ratio in posterior brain regions. Standard imaging procedures were used (TR, 2 s; TE, 70 ms; flip angle, 90°; 180° offset, 25 ms) and have been described²⁴. Twelve 6-mm-thick near-coronal slices were orientated parallel to the brainstem and covered the occipital lobe as well as the posterior portions of the temporal and parietal lobes; 168 functional images were collected for each slice in each scan.

Data analysis. For each subject, data from all the runs in each experiment were averaged together. The time course of MR signal intensity was extracted from each subject's PPA (averaging over all voxels within the ROI). The data from experiment 2 were used to define the regions of interest (ROIs) for experiment 1, the data from experiment 1 were used to define the ROIs for experiment 2, and the data from an additional experiment not reported here were used to define the ROIs for experiment 3. ROIs included all contiguous voxels in the collateral sulcus region for which a Kolmogorov-Smirnov test revealed higher levels of activation at the significance level of $P < 10^{-4}$ for scenes compared to the average response to faces and objects. The per cent signal change in the PPA ROI was calculated for each subject, experiment, stimulus condition, and task (incorporating an estimated 6-s haemodynamic lag and 4-s interepoch buffer of data which was omitted from the analysis), using the average signal intensity during fixation epochs for the same subject, experiment and task as a baseline²⁴. ANOVAs across subjects were run on the average per cent signal change in each of the conditions in each experiment. Because data were analysed within independently defined ROIs, no correction for multiple voxelwise comparisons was made. An analysis of possible laterality effects using separate left versus right hemisphere ROIs for experiment 2 found no main effect of laterality or interaction with any other variable (all F values were <1); therefore laterality was excluded as a factor from all other analyses.

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- O'Keefe, J. & Nadel, L. *The Hippocampus as a Cognitive Map* (Oxford University Press, Oxford, 1978).
- Aguirre, G. K., Detre, J. A., Alsop, D. C. & D'Esposito, M. The parahippocampus subserves topographical learning in man. *Cerebr. Cort.* **6**, 823–829 (1996).
- Aguirre, G. K. & D'Esposito, M. Environmental knowledge is subserved by separable dorsal/ventral neural areas. *J. Neurosci.* **17**, 2512–2518 (1997).
- Maguire, E. A., Frackowiak, R. S. J. & Frith, C. D. Learning to find your way: a role for the human hippocampal region. *Proc. R. Soc. Lond. B* **263**, 1745–1750 (1996).
- Maguire, E. A., Frackowiak, R. S. J. & Frith, C. D. Recalling routes around London: activation of the right hippocampus in taxi drivers. *J. Neurosci.* **17**, 7103–7110 (1997).
- Rolls, E. T., Robertson, R. G. & Georges-Francois, P. Spatial view cells in the primate hippocampus. *Eur. J. Neurosci.* **9**, 1789–1794 (1997).
- Stern, C. E. *et al.* The hippocampal formation participates in novel picture encoding: evidence from functional magnetic resonance imaging. *Proc. Natl. Acad. Sci. USA* **93**, 8660–8665 (1996).
- Gabrieli, J. D. E., Brewer, J. B., Desmond, J. E. & Glover, G. H. Separate neural bases of two fundamental memory processes in the human medial temporal lobe. *Science* **276**, 264–266 (1997).
- Gaffan, D. Scene-specific memory for objects: a model of episodic memory impairment in monkeys with fornix transection. *J. Cogn. Neurosci.* **6**, 305–320 (1994).
- Aguirre, G. K., Zarahn, E. & D'Esposito, M. Studies of the neuro-anatomical components of topographical representation. *Proc. Natl. Acad. Sci. USA* **95**, 839–846 (1998).
- Ishai, A., Ungerleider, L. G., Martin, A., Maisog, J. M. & Haxby, J. V. fMRI reveals differential activation in the ventral object vision pathway during the perception of faces, houses, and chairs. *NeuroImage* **5**, S149 (1997).
- Hermer, L. & Spelke, E. S. A geometric process for spatial reorientation in young children. *Nature* **370**, 57–59 (1994).
- Hermer, L. & Spelke, E. Modularity and development: the case of spatial reorientation. *Cognition* **61**, 195–232 (1996).
- Cheng, K. A purely geometric module in the rat's spatial representation. *Cognition* **23**, 149–178 (1986).
- Margules, J. & Gallistel, C. R. Heading in the rat: determination by environmental shape. *Anim. Learn. Behav.* **16**, 404–410 (1988).
- Gallistel, C. R. *The Organization of Learning* (MIT Press, Cambridge, MA, 1990).
- Biegler, R. & Morris, R. G. M. Landmark stability is a prerequisite for spatial but not discrimination learning. *Nature* **361**, 631–633 (1993).
- Knierem, J. J., Kudrimoti, H. S. & McNaughton, B. L. Place cells, head direction cells, and the learning of landmark stability. *J. Neurosci.* **15**, 1648–1659 (1995).
- Fodor, J. A. *The Modularity of Mind* (MIT Press, Cambridge, MA, 1983).
- Habib, M. & Sirigu, A. Pure topographical disorientation: a definition and anatomical basis. *Cortex* **23**, 73–85 (1987).
- Landis, T., Cummings, J. L., Benson, D. F. & Palmer, E. P. Loss of topographic familiarity: an environmental agnosia. *Arch. Neurol.* **43**, 132–136 (1986).
- Maguire, E. A., Burke, T., Phillips, J. & Staunton, H. Topographical disorientation following unilateral temporal lobe lesions in humans. *Neuropsychologia* **34**, 994–1001 (1996).
- Bohbot, V. *et al.* Spatial memory deficits in patients with lesions to the right hippocampus and to the right parahippocampal cortex. *Neuropsychologia* (in the press).

24. Kanwisher, N., McDermott, J. & Chun, M. M. The fusiform face area: a module in human extrastriate cortex specialized for face perception. *J. Neurosci.* **17**, 4302–4311 (1997).

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Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice

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L-glutamate, the neurotransmitter of the majority of excitatory synapses in the brain, acts on three classes of ionotropic receptors: NMDA (N-methyl-D-aspartate), AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and kainate receptors. Little is known about the physiological role of kainate receptors because in many experimental situations it is not possible to distinguish them from AMPA receptors^{1,2}. Mice with disrupted kainate receptor genes enable the study of the specific role of kainate receptors in synaptic transmission as well as in the neurotoxic effects of kainate. We have now generated mutant mice lacking the kainate-receptor subunit GluR6. The hippocampal neurons in the CA3 region of these mutant mice are much less sensitive to kainate. In addition, a postsynaptic kainate current evoked in CA3 neurons by a train of stimulation of the mossy fibre system is absent in the mutant^{3,4}. We find that GluR6-deficient mice are less susceptible to systemic administration of kainate, as judged by onset of seizures and by the activation of immediate early genes in the hippocampus. Our results indicate that kainate receptors containing the GluR6 subunit are important in synaptic transmission as well as in the epileptogenic effects of kainate.

We disrupted the GluR6 (or *Grik2*) gene⁵ by homologous recombination (Fig. 1a, b). GluR6^{-/-} mice did not differ from their littermates in breeding and general health status, except for a slight reduction in body weight (GluR6^{+/-}: 29.3 $\pm$ 0.8 g, n = 10; GluR6^{-/-}: 26.2 $\pm$ 0.6 g, n = 16, P < 0.05). In regions known to express the GluR6 gene, that is, the granule cell layer of the cerebellum or CA3 and dentate gyrus of the hippocampus, no GluR6 messenger RNA could be detected by *in situ* hybridization using a probe recognizing the 3'-end of the mRNA (Fig. 2a). Immunoblot analysis using an anti-GluR6/7 antibody⁶ on membrane extracts from wild-type hippocampus showed a strong band corresponding to a relative molecular mass of 115,000 (M_r 115K)

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(Fig. 2b), which is reduced in intensity in GluR6^{-/+} mice and absent in GluR6^{-/-} mice. The distribution of high-affinity kainate-binding sites was studied using [³H]kainic acid (12 nM) for receptor autoradiography. The intense ³H labelling in the CA3 region and dentate gyrus of wild-type mice was absent from the same region in GluR6^{-/-} mutant mice (Fig. 2c). ³H labelling was also strongly decreased in the granule cell layer of the cerebellum and in the neostriatum (data not shown), but not in the deeper lamina of the neocortex (Fig. 2c) or in the molecular layer of the cerebellum. Expression of the kainate receptor subunit KA-1 mRNA, which is colocalized with high-affinity kainate-binding sites in the hippocampus⁷, was unchanged as determined by *in situ* hybridization (data not shown).

We analysed wild-type and mutant mice for the presence of functional kainate receptors in CA3 neurons of the hippocampus. These neurons are sensitive to submicromolar concentrations of kainate⁸, although it has not been clear whether this high sensitivity is due to an action of kainate on AMPA receptors or on kainate receptors. We compared the effects of bath-applied kainate on CA3 neurons in slices from wild-type and GluR6^{-/-} mice. In wild-type mice, kainate evoked sustained inward currents at 300 nM (amplitude, 54 ± 14 pA; *n* = 5) (Fig. 3a). In GluR6^{-/-} mice, however, kainate failed to activate inward currents at concentrations as high as 3 μM, a concentration at which large whole-cell currents are readily recorded in wild-type mice (Fig. 3b) (678 ± 128 pA; *n* = 5 in GluR6^{+/+} mice; <10 pA, *n* = 5 in GluR6^{-/-} mice). Dose-response curves established for bath-applied kainate (0.3–30 μM) indicate a reduced sensitivity of CA3 neurons to kainate in GluR6^{-/-} mice (Fig. 3c). It has been shown that kainate activates an inward current in CA3 neurons in the presence of GYKI 53655 (refs 3, 4), a highly selective AMPA-receptor antagonist. In the presence of GYKI 53655 (30 μM), an inward current was activated by 3 μM kainate in wild-type mice (*n* = 3) but not by 30 μM kainate in GluR6^{-/-} mutant mice (*n* = 3). This latter result indicates that the effect of higher concentrations of kainate in GluR6^{-/-} mice, in the absence of GYKI 53655, is mediated by activation of AMPA receptors but not of kainate receptors. Taken together, these data show that at kainate concentrations of up to a few μM, only kainate receptors are activated by kainate in CA3 neurons and that these receptors contain the GluR6 subunit.

We next tested whether GluR6 was involved in the slow synaptic current evoked in CA3 neurons by train stimulation of the mossy fibre pathway, referred to as kainate excitatory postsynaptic current (EPSC)^{3,4}. As this synaptic current was recorded in the presence of picrotoxin, DL-AP5 (2-amino-5-phosphonovaleric acid) and GYKI 53655, but not in the presence of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an antagonist at both AMPA and kainate receptors, these studies concluded that it was mediated by kainate receptors. We recorded synaptic currents evoked in CA3 neurons by stimulation of the mossy fibre pathway in the presence of DL-AP5 (50 μM) and picrotoxin (100 μM) in order to block the NMDA and GABA_A (γ-aminobutyric acid) receptors, respectively. Mossy fibre synapses onto CA3 neurons show a prominent paired-pulse facilitation (PPF), seen as an increase in the size of the synaptic response when a synapse is activated twice at short intervals^{9,10}. PPF (40-ms interval) was similar in GluR6^{+/+} (4.3 ± 0.6, *n* = 14) and GluR6^{-/-} (4.4 ± 0.7, *n* = 10) mice (Fig. 4). In addition, mossy fibre EPSCs in wild-type and GluR6-deficient mice were rapidly antagonized by bath application of LCCG-I (10 μM) (GluR6^{+/+}, *n* = 7; GluR6^{-/-}, *n* = 6) (Fig. 4), an agonist at group II metabotropic glutamate receptors which suppresses transmission at mossy fibre synapses¹¹. These results indicate that the mossy fibre synapse maintains normal function in GluR6-deficient mice. Bath application of GYKI 53655 (30 μM) suppressed mossy fibre EPSCs (Fig. 4), as well as spontaneous EPSCs (data not shown). However, in the wild type, a synaptic response was restored when a train of stimuli (typically 3 pulses at 5-ms intervals) was given to the mossy fibres (Fig. 4) (87 ± 9 pA, range 45–128 pA, *n* = 10). Increasing the

number of stimuli in the train and the intensity of the stimulus increased the amplitude of the synaptic currents. In the presence of GYKI 53655 and using the same stimulation protocol, no kainate EPSC was observed in GluR6-deficient mice (Fig. 4), indicating that kainate receptors containing the GluR6 subunit participate in synaptic transmission in the mossy fibre pathway.

Behavioural analysis of GluR6^{-/-} mice did not reveal any significant deficits in several sensorimotor tests, including walk initiation, limb posture, visual placement, bridge crossing, negative geotaxis and muscle strength (data not shown). A rotarod test showed that

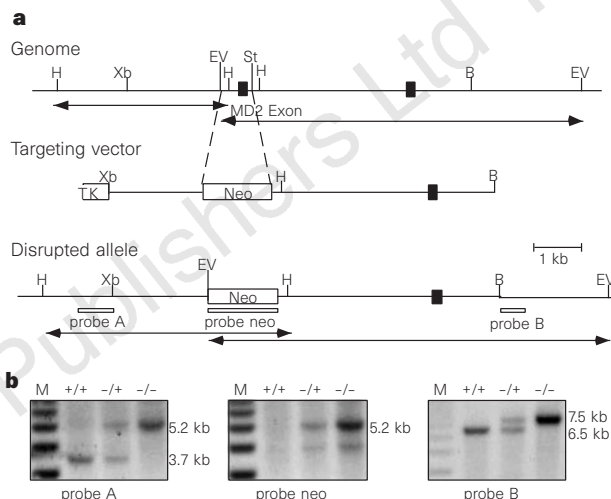


Figure 1 Disruption of the GluR6 gene by homologous recombination. **a**, top line, Structure of the genomic region of the GluR6 gene around the exon coding for the MD2; middle line, the targeting vector contained 2.0 kb and 4.7 kb, respectively, of 5' and 3' homologous flanking DNA and carried a 0.7 kb deletion including the exon coding for MD2; bottom line, genomic structure of the mutant allele generated by homologous recombination. Sequences used as probes in Southern blot analyses are shown. Restriction enzyme sites are indicated. H, *Hind*III; Xb, *Xba*I; EV, *Eco*RV; St, *Stu*I; B, *Bam*HI. **b**, Southern blot analysis of mouse genomic tail DNA from wild-type (+/+), heterozygous (-/+) and homozygous (-/-) mutant mice using probes A or neo on *Hind*III-digested DNA, and probe B on *Eco*RV-digested DNA.

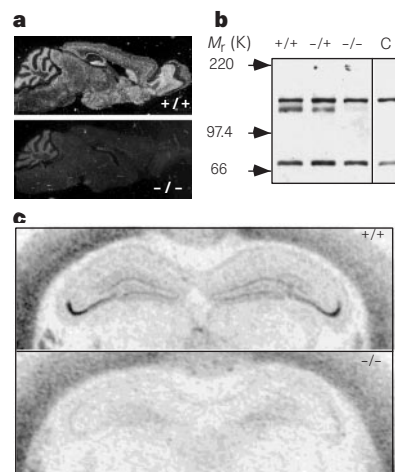


Figure 2 Analysis of GluR6 mutant mice by *in situ* hybridization, immunoblotting and receptor autoradiography. **a**, *In situ* hybridization of parasagittal sections using ³³P-labelled specific RNA probes for GluR6 mRNA. **b**, Immunoblot of hippocampal membrane extracts using anti-GluR6/7 antibodies. The top and bottom band represent nonspecific crossreactivity of the secondary antibody, as shown in the control lane C using wild-type extract without primary antibody. **c**, Receptor autoradiography using [³H]kainate on coronal sections from wild-type and mutant mice.

there was no significant difference between different genotypes, as judged by an increase in latency before falling from a rotating rod (3 trials per day over 3 days; $P < 0.0001$), indicating that sensory motor learning ability was normal. These results suggest that there is no obvious deficit attributable to altered cerebellar function. Analysis of general locomotor activity indicated that GluR6^{-/-} mice were less active (average beam breaks in a 5-min interval; 166.3 ± 5.2 for GluR6^{+/+} mice, $n = 10$; 112.6 ± 3.0 for GluR6^{-/-} mice, $n = 16$, $P < 0.01$) and that the activity of both groups decreased over the two days ($P < 0.0001$). In a Morris watermaze test (4 trials per day

over 9 days), a comparable learning ability was demonstrated by both wild-type and mutant mice, as judged by reduction in the time taken to find a hidden platform (comparing days 1 and 9, wild-type mice took 37.3 ± 1.2 s and 15.5 ± 2.4 s ($n = 10$); mutant mice took 34.8 ± 1.3 s and 20.2 ± 3.0 s ($n = 16$), $P < 0.0001$, respectively). However, more extensive training and testing is needed to define the nature of the learning strategy.

Administration of kainic acid to rodents causes a well characterized seizure syndrome that is associated with excitotoxic neurodegeneration in selected populations of neurons located in the

Figure 3 Reduced sensitivity of CA3 neurons to bath application of kainate in GluR6^{-/-} mice. **a, b**, Whole-cell currents recorded in CA3 neurons from a GluR6^{+/+} **a** and a GluR6^{-/-} **b** mouse. Neurons were voltage-clamped at -70 mV. Kainate was added to the perfusion medium containing tetrodotoxin (TTX) (500 nM), DL-AP5 (50 μ M) and picrotoxin (100 μ M) at the concentrations indicated on the left of the traces for 2 min (white box). For the bottom traces, GYKI 53655 (50 μ M) was added to the perfusion medium 1–2 min before kainate. **c**, Amplitude of kainate-activated whole-cell inward currents as a function of kainate concentration. All values are mean $\pm$ s.e.m. of five values taken from five different cells from three GluR6^{+/+} and three GluR6^{-/-} mice. At each kainate concentration, the difference between wild-type and GluR6^{-/-} values was significant ($P < 0.001$). Scale bars, 100 pA; holding potential, -70 mV.

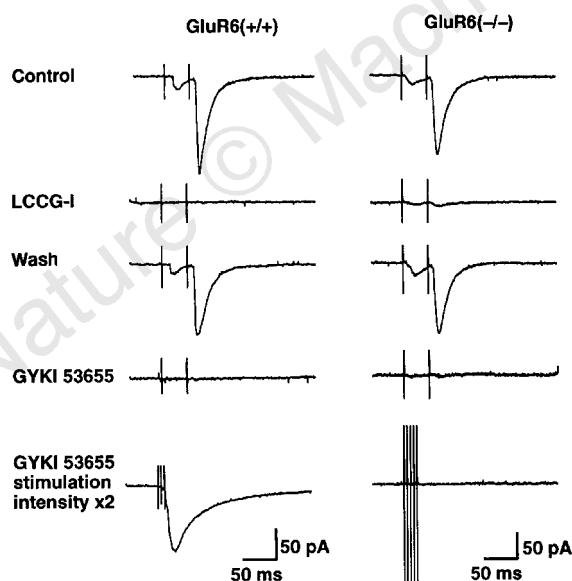
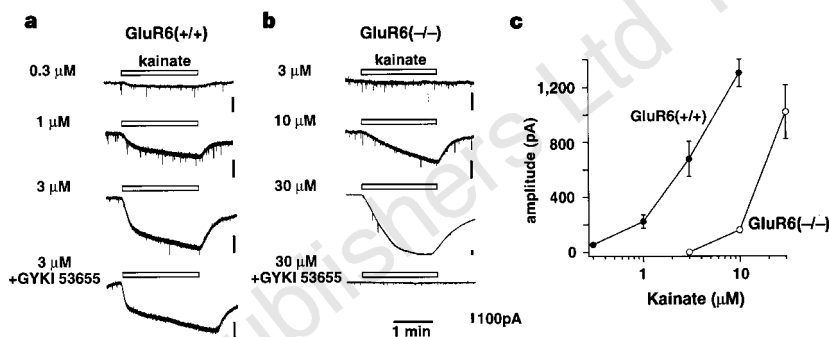


Figure 4 Mossy fibre kainate EPSC cannot be detected in GluR6^{-/-} mice. Whole-cell synaptic currents evoked by stimulation of the mossy fibre in GluR6^{+/+} and GluR6^{-/-} mice. Each trace represents the average of 10 consecutive recordings. The magnitude of the paired-pulse facilitation (40 ms interval) was similar in normal and mutant mice (GluR6^{+/+} 4.3 ± 0.6 , $n = 14$; GluR6^{-/-} 4.4 ± 0.7 , $n = 10$). LCCG-I caused a reversible inhibition of mossy fibre synaptic transmission in both wild-type (7 neurons from 5 mice) and GluR6^{-/-} mice (6 neurons from 3 mice). GYKI 53655 (30 μ M) blocked AMPA-receptor-mediated EPSCs in both wild-type (10 neurons, 7 mice) and GluR6^{-/-} mice (7 neurons, 4 mice). In wild-type mice, stimulating the mossy fibre system with a burst of three pulses (200 Hz) restored a slow synaptic current, the amplitude of which could be increased by doubling the stimulation intensity. Holding potential: -70 mV.

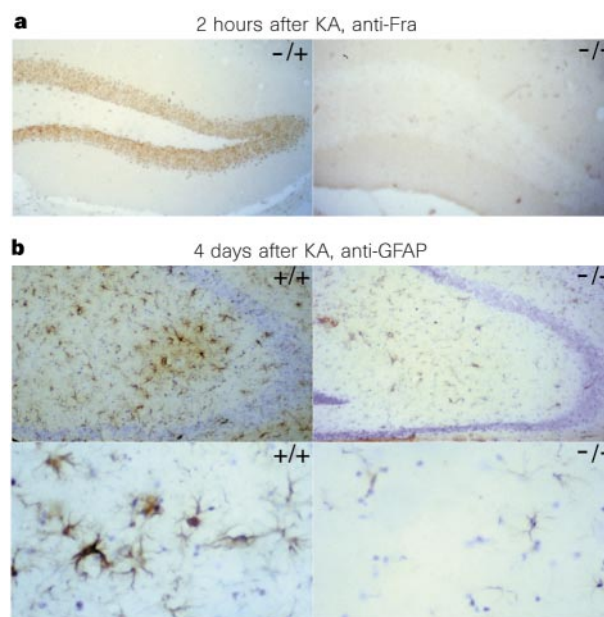


Figure 5 Analysis of immediate-early gene (IEG) induction and astrogliosis in kainate-treated mice. **a**, Immunostaining for IEG induction in the dentate gyrus of GluR6^{+/+} and GluR6^{-/-} mice 2 hours after systemic application of 20 mg kg⁻¹ kainic acid using anti-Fra antibodies. **b**, Analysis of astrogliosis in the CA3 and CA2 region of the hippocampus in wild-type and GluR6^{-/-} animals 4 days after systemic application of 20 mg kg⁻¹ kainic acid using anti-GFAP antibodies.

Table 1 Seizure severity and latency of onset after systemic administration of kainate

Genotype	Kainate (mg kg ⁻¹)	Seizure (no. of animals with seizure/total)	Seizure index (a.u. $\pm$ s.e.)	Latency (min $\pm$ s.e.)
GluR6 ^{+/+}	20	6/6	3.7 $\pm$ 0.6	14.2 $\pm$ 1.7
GluR6 ^{-/-}	20	11/11	3.4 $\pm$ 0.4	18.2 $\pm$ 1.5
GluR6 ^{-/-}	20	1*/17	(0.06)*	(5.0)*
129Sv	20	7/9	1.6 $\pm$ 0.3	28.7 $\pm$ 3.8
C57BL/6	20	10/10	3.2 $\pm$ 0.4	17.5 $\pm$ 2.1
GluR6 ^{+/+}	30	7/8	4.0 $\pm$ 0.7	8.3 $\pm$ 1.1
GluR6 ^{-/-}	30	5/6	4.2 $\pm$ 0.8	8.6 $\pm$ 1.5

* One animal had a single seizure after 5 min; seizure index and latency are given for this episode.
a.u.: arbitrary units (see Methods).

hippocampus^{12,13}. Because it has been hard to define a specific role for kainate receptors in excitotoxicity, we administered kainate intraperitoneally at 20 mg kg⁻¹ to control mice and to GluR6-deficient mice. The latency before seizure onset and the number and severity of seizures were recorded for two hours after injection and a seizure index (SI) was calculated (see Methods). GluR6^{+/+} mice (6/6, SI: 3.7 $\pm$ 0.6) and GluR6^{-/-} mice (11/11, SI: 3.4 $\pm$ 0.4) all suffered seizures, whereas GluR6^{-/-} mice were almost all protected against seizure (16/17 mice) (Table 1). At higher doses (30 mg kg⁻¹) of kainic acid, GluR6^{-/-} mice also had seizures (5/6). As susceptibility to kainate depends on the genetic background of the mice¹⁴, we tested the susceptibility to kainate-induced seizures of the two parental mouse strains, in addition to testing GluR6^{-/-} and control mice in the hybrid background. 129Sv mice showed a significant⁷ lower seizure index (1.6 $\pm$ 0.3), but seven out of nine mice had seizures at 20 mg kg⁻¹ doses of kainate. The susceptibility to seizure of the C57BL/6 mouse strain was comparable to the hybrid strain (10/10, SI: 3.4 $\pm$ 0.4) (Table 1), so it is unlikely that the genetic background accounts for the differences observed between wild-type and mutant mice.

Activation of immediate early genes, in particular of the proto-oncogene *c-fos*¹⁵ and its related proteins, in regions susceptible to kainate injection, precedes kainate-induced neuronal damage and is a harbinger of death¹⁶. Two hours after kainate treatment, all GluR6^{+/+} and GluR6^{-/-} mice (9 mice analysed) showed a marked increase in immunoreactivity to c-Fos and Fos-related antigens (Fra)¹⁷ in the dentate gyrus, and to a lesser extent in CA3 of the hippocampus (4 out of 6). GluR6^{-/-} mice (10 out of 11) did not show any detectable induction of Fra (Fig. 5a). Neurodegeneration was assessed by staining brain sections with anti-glial fibrillary acidic protein (GFAP) antibodies in mice 4 days after kainate injection. Wild-type and heterozygous mice ($n = 2$) showed severe astrogliosis with strong induction of GFAP, especially in the hippocampus, which is indicative of neuronal degeneration. In contrast, GluR6^{-/-} mice ($n = 2$) showed only basal levels of expression (Fig. 5b).

This study indicates that kainate receptors assembled with the GluR6 subunit are important for both the sensitivity of CA3 neurons to kainate and for synaptic transmission at the mossy fibre-CA3 synapse. Analysis of mice mutant for the kainate receptor, in parallel with studies using pharmacological agents¹⁷⁻¹⁹, will help to establish the role of kainate receptors in synaptic transmission. Our results demonstrate a critical role for kainate receptors, as opposed to AMPA receptors, in kainate-induced seizures and excitotoxic neuronal death in the hippocampus. Kainate-induced seizures are a model for human temporal lobe epilepsy, so drugs that interfere with GluR6 kainate-receptor function may be useful for treating seizures and other neurological diseases involving excitotoxicity. □

Methods

Generation of GluR6-deficient mice. The targeting construct consisted of a 7.4-kb *XbaI/BamHI* restriction fragment from a 129Sv mouse gene (Fig. 1a) in

which a PGK-*neo*^R cassette replaces the *EcoRV/StuI* fragment (687 bp) that overlaps MD2. Transfection and selection procedures into W9.5 ES cells have been described²⁰. Positive clones (4/578) were identified by Southern blotting and injected into C57BL/6 blastocysts. One chimera transmitted the mutation through the germ line and was backcrossed to C57BL/6 for one generation; the resulting C57BL/6 $\times$ 129Sv (F₂ generation) were intercrossed to produce GluR6^{-/-} mice at a mendelian ratio (25%, $n = 170$).

Histological procedures. *In situ* hybridization was performed as described²¹ with ³³P-labelled RNA probes (GluR6 3'-probe: nucleotides 2,471–3,017; KA-1 5'-probe: nucleotides 1–215; ref. 21). Membrane-protein extracts from mouse hippocampus were prepared as before²²; immunoblots were probed with a rabbit polyclonal antiserum recognizing GluR6/7 (Upstate Biotechnology). Autoradiography with [³H]kainic acid was done by incubating 20- μ m cryostat sections with 12 nM [³H]kainic acid (50 Ci mmol⁻¹)²³. For immunocytochemistry, an antiserum recognizing c-Fos and other Fos-related antigens (1:1,000 dilution)²⁴ and an anti-GFAP monoclonal antibody (Boehringer-Mannheim) were used as primary antibodies.

Electrophysiology. Whole-cell patch-clamp recordings were made at room temperature from visually identified CA3 neurons in hippocampal slices (400 μ m) from 14–31-day-old mice. Slices were bathed in an oxygenated solution composed of (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 25 glucose, pH 7.4. When electrical stimulation was performed, the concentration of CaCl₂ and MgCl₂ increased to 4 mM. Patch electrodes (2.3–3.2 M Ω ; access resistance 6–12 M Ω) were filled with (in mM): 140 CsCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 HEPES, 2 Na-ATP, pH 7.3. EPSCs were evoked by low-frequency (0.3–0.5 Hz) stimulation of the mossy fibre pathway using 20- μ s pulses delivered through bipolar tungsten electrodes placed in the granule cell layer.

Behavioural analysis. All mice (GluR6^{+/+}, $n = 10$; GluR6^{-/-}, $n = 16$) used were 3–6-month-old male F₃ C57BL/6 $\times$ 129Sv hybrids, obtained by mating heterozygous F₂ mice. All behavioural testing and data collection were done by an investigator blinded to the genotype of the mice. For rotarod motor learning, mice were trained (3 trials per day for 3 days) by placing them on an accelerating rod (6–65 r.p.m., within 3 min). The latency time taken to fall from the rod was recorded. Locomotor activity of mice placed in square chambers (61 cm $\times$ 61 cm) was automatically recorded (5-min time bins) for 40 min on two days. The hidden-platform watermaze test has been described²⁵.

Kainate administration. C57BL/6 $\times$ 129Sv hybrid mice (GluR6^{+/+}, $n = 6$; GluR6^{-/-}, $n = 19$; GluR6^{-/-}, $n = 23$) were obtained by mating heterozygous F₂ mice (aged 6–14 months). After intraperitoneal administration of kainic acid, mice were monitored continuously for 2 h for onset and extent of seizures. Seizure severity was rated according to the arbitrary scale: 0, no seizure; 1, 1 seizure; 2, 2–5 seizures; 3, 5–10 seizures; 4, >10 seizures, or severe tonic clonic seizure; 5, death within 2 h. The seizure index (SI) was calculated by averaging the points for seizure severity in a given group.

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- Bettler, B. & Mulle, C. AMPA and kainate receptors. *Neuropharmacology* **34**, 123–139 (1995).
- Lerma, J., Morales, M., Vicente, M. A. & Herreras, O. Glutamate receptors of the kainate type and synaptic transmission. *Trends Neurosci.* **20**, 9–12 (1997).
- Castillo, P. E., Malenka, R. C. & Nicoll, R. A. Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. *Nature* **388**, 182–186 (1997).
- Vignes, M. & Collingridge, G. L. The synaptic activation of kainate receptors. *Nature* **388**, 179–182 (1997).
- Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I. & Heinemann, S. Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature* **351**, 745–748 (1991).
- Raymond, L. A., Blackstone, C. D. & Huganir, R. L. Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. *Nature* **361**, 637–641 (1993).
- Werner, P., Voigt, M., Keinänen, K., Wisden, W. & Seeburg, P. H. Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. *Nature* **351**, 742–744 (1991).
- Robinson, J. H. & Deadwyler, S. A. Kainic acid produces depolarization of CA3 pyramidal cells in the *in vitro* hippocampal slices. *Brain Res.* **221**, 117–127 (1981).
- Zucker, R. Short-term synaptic plasticity. *Annu. Rev. Neurosci.* **12**, 13–31 (1989).
- Salin, P., Scanziani, M., Malenka, R. C. & Nicoll, R. Distinct short-term plasticity at two excitatory synapses in the hippocampus. *Proc. Natl Acad. Sci. USA* **93**, 13304–13309 (1996).
- Kamiya, H., Shinozaki, H. & Yamamoto, C. Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fibre synapse. *J. Physiol.* **493**, 447–455 (1996).
- Ben-Ari, Y. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* **14**, 375–403 (1985).
- Nadler, J. Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells. *Nature* **271**, 676–677 (1978).
- Schauwecker, P. & Steward, O. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. *Proc. Natl Acad. Sci. USA* **94**, 4103–4108 (1997).
- Le Gal La Salle, G. Long-lasting and sequential increase in c-fos oncoprotein expression in kainic-acid induced status epilepticus. *Neurosci. Lett.* **88**, 127–130 (1988).

16. Smye, R. J. *et al.* Continuous *c-fos* expression precedes programmed cell death *in vivo*. *Nature* **363**, 166–169 (1993) (erratum, *ibid.* **365**, 279; 1993).
17. Chittajallu, R. *et al.* Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. *Nature* **379**, 78–81 (1996).
18. Rodriguez-Moreno, A., Herrera, O. & Lerma, J. Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. *Neuron* **19**, 893–901 (1997).
19. Clarke, V. R. J. *et al.* A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. *Nature* **389**, 599–603 (1997).
20. Vetter, D. E. *et al.* Inner ear defects induced by null mutation of the *isk* gene. *Neuron* **17**, 1251–1264 (1996).
21. Bischoff, S., Barhanin, J., Bettler, B., Mulle, C. & Heinemann, S. Spatial distribution of kainate receptor subunit mRNA in the mouse basal ganglia and ventral mesencephalon. *J. Comp. Neurol.* **379**, 541–562 (1997).
22. Forrest, D. *et al.* Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. *Neuron* **13**, 325–338 (1994).
23. Monaghan, D. & Cotman, C. The distribution of [³H]-kainic acid binding sites in rat CNS as determined by autoradiography. *Brain Res.* **252**, 91–100 (1982).
24. Young, S., Porrino, L. & Iadarola, M. Cocaine induces striatal *c-Fos*-immunoreactive proteins via dopaminergic D1 receptors. *Proc. Natl Acad. Sci. USA* **88**, 1291–1295 (1991).
25. Kempermann, G., Kuhn, H. G. & Gage, F. H. More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386**, 493–495 (1997).

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Mutations in the *parkin* gene cause autosomal recessive juvenile parkinsonism

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Parkinson's disease is a common neurodegenerative disease with complex clinical features¹. Autosomal recessive juvenile parkinsonism (AR-JP)^{2,3} maps to the long arm of chromosome 6 (6q25.2-q27) and is linked strongly to the markers *D6S305* and *D6S253* (ref. 4); the former is deleted in one Japanese AR-JP patient⁵. By positional cloning within this microdeletion, we have now isolated a complementary DNA clone of 2,960 base pairs with a 1,395-base-pair open reading frame, encoding a protein of 465 amino acids with moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxy terminus. The gene spans more than 500 kilobases and has 12 exons, five of which (exons 3–7) are deleted in the patient. Four other AR-JP patients from three unrelated families have a deletion affecting exon 4 alone. A 4.5-kilobase transcript that is expressed in many human tissues but is abundant in the brain, including the substantia nigra, is shorter in brain tissue from one of the groups of exon-4-deleted patients. Mutations in the newly identified gene appear to be responsible for the pathogenesis of AR-JP, and we have therefore named the protein product 'Parkin'.

To investigate a small chromosomal deletion in a Japanese AR-JP patient, we first used screening by polymerase chain reaction (PCR) with a set of amplimers for *D6S305* to isolate two clones, KB761D4

and KB430C4 (approximate insert size 110 kilobases (kb) each) from a Keio human BAC (bacterial artificial chromosome) library⁶. Using exon trapping with these two BAC DNAs, we unexpectedly found only one putative exon (designated J-17) of 137 base pairs (bp). Amplification by PCR of the patient's DNA using two sets of amplimers, created from the J-17 sequence and its flanking regions, revealed that the patient has a deletion of J-17 (Fig. 1b) in addition to one in *D6S305*.

We screened human fetal brain and skeletal muscle cDNA libraries with J-17 as an initial probe and then with the cDNAs, once isolated, as secondary probes. Seven cDNA clones were obtained and sequenced. The aligned sequence of 2,960 bp revealed an open reading frame of 1,395 bp (nucleotides 102–1,496), which encodes a protein of 465 amino acids with a relative molecular mass (*M_r*) 51,652 (Fig. 2). Four of seven cDNA clones lost a small sequence of 84 bp (nucleotides 636–719: amino-acid residues 179–206) in frame, implying that alternative splicing was involved (Fig. 2).

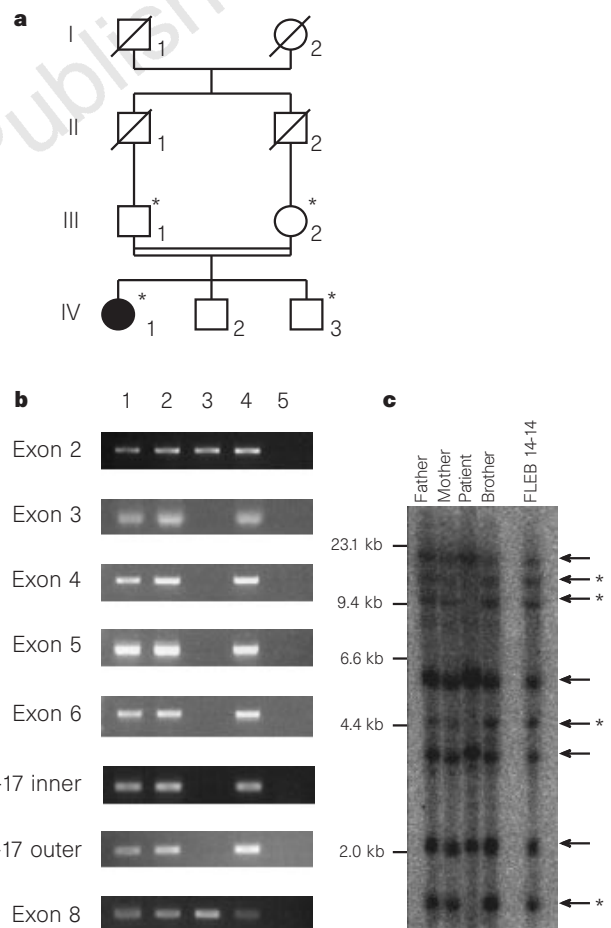


Figure 1 Genetic studies of family 1. **a**, Pedigree of family 1. A filled circle represents the affected woman (IV-1). Open circles (women) and open squares (men) represent normal individuals. Asterisks indicate family members whose DNA samples were analysed. Symbols with lines through them represent the deceased. **b**, PCR analysis of family 1. Genomic DNA was processed for PCR amplification with amplimers for exons 2, 3, 4, 5, 6, J-17 inner, J-17 outer and 8. Lane 1, father (III-1); lane 2, mother (III-2); lane 3, patient (IV-1); lane 4, brother (IV-3); lane 5, no template DNA. **c**, Genomic Southern blot analysis of the family 1 members using the cDNA clone including open reading frame as probe. At least eight *EcoRI* fragments were detected in the DNA of healthy members, but only four *EcoRI* fragments were found in the patient DNA. Asterisks indicate the lost bands in the patients. The lane of FLEB 14-14 contains *EcoRI* digests of genomic DNA from human pre-pro B-cell line FLEB 14-14 (ref. 6).

Figure 2 Amino-acid sequence of Parkin protein. The portion with homology to ubiquitin is boxed. The 28 amino acid residues that are alternatively spliced are indicated by bold underlining. Cysteine and histidine residues composing the RING-finger-like motif are in bold and are underlined. Note that DNA sequence analysis of all the individuals examined in this study indicated a C to T transition at bp 768 as compared to the cDNA sequence, inducing a proline to serine transition at amino-acid position 223.

Parkin	1	MIVFVRFNSSHGFPVEVDS	50
UBIQUITIN HUMAN	1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPPDOORLIFAGKQL	50
UBIQUITIN YEAST	1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPPDOORLIFAGKQL	50
UBIQUITIN SOYBEAN	1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPPDOORLIFAGKQL	50

Parkin	51	RNDWTVQNCDDLQQSIVHIVQRPWRK	76
UBIQUITIN HUMAN	51	EDGRITLSDYNIQKESTLHLVLR	76
UBIQUITIN YEAST	51	EDGRITLSDYNIQKESTLHLVLR	76
UBIQUITIN SOYBEAN	51	EDGRITLADYNIQKESTLHLVLR	76

The deduced amino-acid sequence of the newly identified protein showed moderate similarity to ubiquitin at the N terminus from Met1 to Lys76 (identities, 32%; positive, 62%) (Fig. 3). The lysine residue at position 48, which is essential for multi-ubiquitin chain formation⁷, is conserved together with its flanking residues. Moreover, the remainder of the Parkin protein is rich in cysteine, and the C terminus has a motif similar to a RING-finger motif^{8,9} (Fig. 2): the sequence of Cys-X₂-Cys-X₉-Cys-X₁-His-X₂-[Cys-X₄-Cys]-X₄-Cys-X₂-Cys at the C terminus of Parkin matches the consensus sequence of the RING-finger apart from at the cysteine residues indicated in brackets. Parkin can therefore be considered as a member of the RING-finger family and/or as a new zinc-finger protein.

We next determined the genomic organization and exon/intron boundary sequences of *parkin*. As initially isolated, two clones contained only one putative exon sequence (J-17), so we also screened the Keio BAC library and a commercial source and obtained an additional 27 clones (see Methods). This extraordinary number of clones implicated in the prospective gene was large, and we estimate *parkin* to be over 500 kb, with large introns (S.A. *et al.*, manuscript in preparation). The sequence of genomic regions corresponding to the entire cDNA was determined by direct sequencing of selected clones (see Methods), and the exon-intron organization and their boundary sequences were established (lodged under accession number AB009973 in the DDBJ database). The *parkin* gene consists of 12 exons, with exon 7 corresponding to the putative exon J-17 obtained by the initial exon trapping. Based on these data, we designed 14 sets of PCR primers to amplify each of 12 exons and determined the size of the expected PCR products.

PCR amplification of the members of family 1 revealed that patient IV-1 lacked five exons (exon 3–7) (Fig. 1a, b), as confirmed by genomic Southern blot analysis (Fig. 1c). Thus, the microdeletion in patient IV-1 is intragenic, and the DNA for seven exons (exons 1, 2 and 8–12) is retained. We analysed two other patients from another unrelated family (family 2, patients II-1 and II-2) and found a deletion in exon 4 in these patients (Fig. 4a and b). This observation was confirmed by PCR analysis with reverse transcription (RT-PCR) of RNA extracted from the brain of patient II-2. The RT-PCR product is 633 bp for a healthy member but only 511 bp for the patient (Fig. 4d). Direct sequencing of the patient PCR product indicated that exon 3 is next to exon 5 with exon 4 missing (Fig. 4c). This exon-4 deletion was found in two additional AR-JP patients from two other unrelated families (data not shown). Thus, the deletion mutation was found in five Japanese AR-JP patients from four independent families. We conclude that the gene encoding the Parkin protein must be responsible for the pathogenesis of AR-JP.

MIVFVRFNSS HGFPVEVDS	TSIFQLKEVV AKRQGV	PADQ LRVIFAGKEL	50
RNDWTVQNCD LDQQSIVHIV QRPWRK	6QEM NATGGDDPRN AAGGCEREP	Q 100	
SLTRVDLSSS VLPGDSVGLA VILHTDSRKD	SPPAGSPAGR SIYNSFYVC	150	
KGPCQRVQPG KLRVQCSTCR QATLTLTQGP	SCWDDVLIPN RMSGECQSPH	200	
CPGTSAEFFF KCGAHTSDK ETPVALHLIA	TNSRNICIT CTDVRSPVLV	250	
FQCNSRHVIC LDCFHLYCVT RLNDRQFVHD	PQLGYSLPCV AGCPNSLIKE	300	
LHHFRILGEE QYNRYQYGA EECVLQMGV	LCPRPGCGAG LLPEPDQRKV	350	
TCEGGNGLGC GFAFCRECKE AYHEGECSAV	FEASGTTTQA YRVDERRAAEQ	400	
ARWEAASKET IKKTTKPCPR CHVPVEKNGG	CMHMKCPQPQ CRLEWCWNC	450	
CEWNRVCMGD HWFDV			

Figure 3 Amino-acid sequence homology between Parkin and the ubiquitin family proteins. Amino-acid sequences of the ubiquitin family proteins were deduced from the cDNA sequences deposited in GenBank and aligned for maximum homology. Boxes indicate sequence identity to Parkin. The lysine residue (K) at position 48 is indicated in bold on a shaded background.

Northern blot analysis, using poly(A)⁺ RNA with J-17 (exon 7) as a probe, showed that a 4.5-kb transcript is expressed in many human tissues, including brain, heart, testis and skeletal muscle (Fig. 5a). In the brain, this gene was expressed in various regions, including substantia nigra (Fig. 5b).

The characteristic clinical symptoms of AR-JP include a susceptibility to dopa-induced dyskinesia and motor fluctuation, in addition to levodopa-responsive parkinsonism^{2,3}. Pathologically, it is characterized by the loss of nigral and locus coeruleus neurons without Lewy body formation¹⁰. Parkinson's disease is probably initiated by a combination of genetic predisposition and environmental factors^{11,12}, which eventually induce mitochondrial respiratory failure and oxidative stress in nigral neurons^{12–14}. A mutation in α -synuclein, a component of the Lewy bodies¹⁵, is linked to the autosomal dominant form of Parkinson's disease¹⁶. We have described a second Parkinson's disease gene which encodes a new protein, 'Parkin', linked to early onset disease that causes neurodegeneration in the substantia nigra without Lewy body formation.

Parkin is similar to the ubiquitin family of proteins, which are involved in the pathogenesis of several neurodegenerative diseases and are a component of the paired helical filaments in Alzheimer's disease^{17–19} and of Lewy bodies in Parkinson's disease^{20–22}. In the paired helical filaments of Alzheimer's disease, ubiquitin is conjugated with tau, but the majority of paired helical filaments remain as mono-ubiquitinated forms, which are not recognized by proteasomes and hence not processed¹⁸. Amyloid β protein inhibits the ubiquitin-dependent proteasome pathway *in vitro*¹⁹. On the other hand, Lewy bodies contain abundant multi-ubiquitin chains²². Incomplete degradation of Lewy-body-associated proteins may cause deposition of multi-ubiquitinated proteins in Lewy bodies²², so ubiquitin or ubiquitin-like proteins may be important in the pathogenesis of Parkinson's disease.

Parkin may function similarly to ubiquitin family members, and its defect in AR-JP may interfere with the ubiquitin-mediated proteolytic pathway leading to the death of nigral neurons^{7,23–25}. Note, however, that no Lewy body formation occurs in AR-JP, in which the *parkin* gene appears to be defective. The ubiquitin portions of several ubiquitin-conjugated proteins also act as chaperones for the remaining portions²⁶, so the ubiquitin-like portion of Parkin may be involved in its functional maturation. Then the C-terminal portion of Parkin containing the RING-finger motif may function as a new zinc-finger protein in the control of cell growth, differentiation and development⁸. Further investigation is necessary to establish the exact physiological function of Parkin and

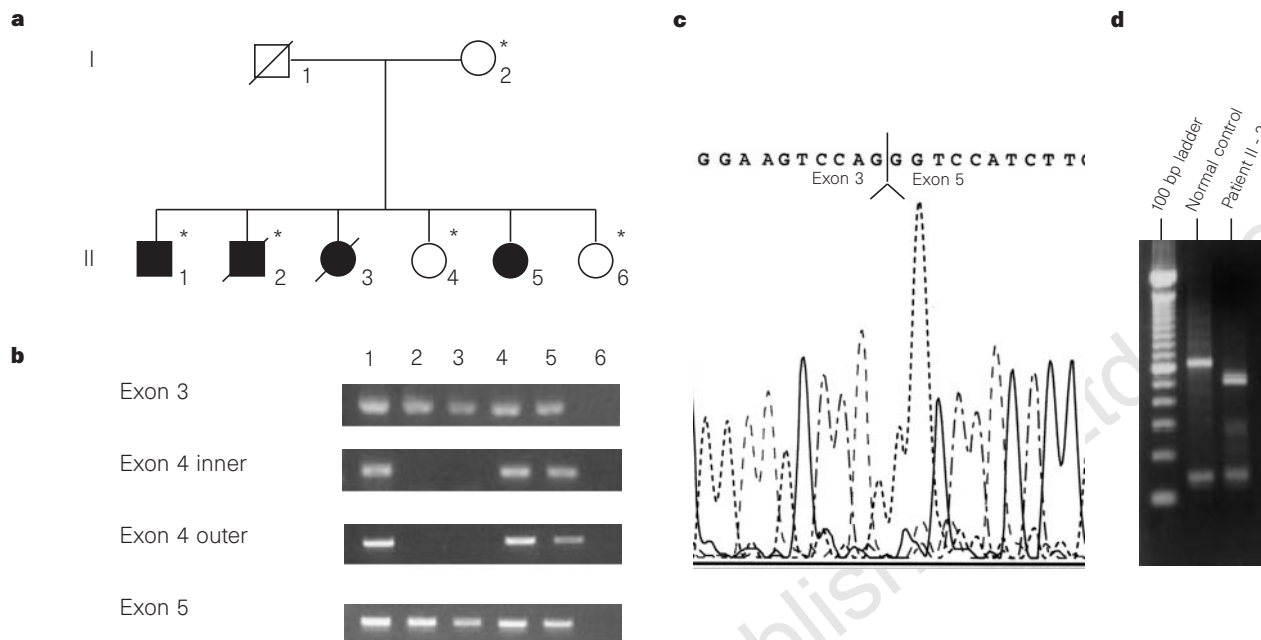


Figure 4 Genetic studies of family 2. **a**, Pedigree of family 2. All affected members are indicated by filled symbols. Asterisks indicate family members whose DNA samples were analysed. **b**, PCR analysis of family 2. Genomic DNA was processed for PCR amplification with amplimers for exon 3, exon 4 inner, exon 4 outer and exon 5. Lane 1, mother (II-2); lane 2, patient (II-1); lane 3, patient (II-2); lane 4, normal sister (II-4); lane 5, normal sister (II-6); lane 6, no template DNA. **c**, Lack of

exon 4 in patient II-2. RT-PCR was performed for total RNA from brain tissue of patient II-2. Exon 3 was next to exon 5, indicating that exon 4 is missing in the patient mRNA. **d**, Analysis of RT-PCR product of patient II-2. The RT-PCR product of the normal control is 633 bp in size (lane 1), whereas the RT-PCR product of patient II-2 is 511 bp (lane 2).

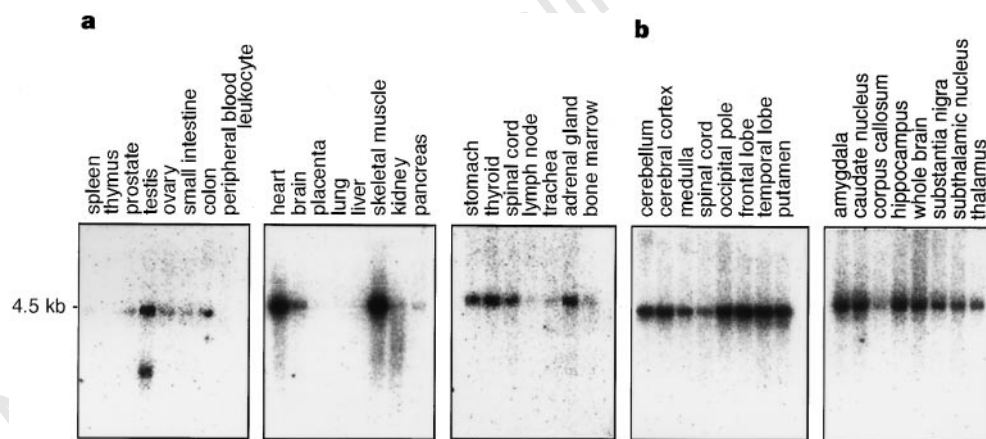


Figure 5 Northern blot analysis of *parkin* gene expression. **a**, Poly(A)⁺ RNA from various human tissues. **b**, Poly(A)⁺ RNAs from various regions of human adult brain.

how the *parkin* gene defect induces selective degeneration of nigral neurons in AR-JP without Lewy body formation. □

Methods

Patients with AR-JP in four families. The affected woman in family 1 (patient IV-1, 43 years old) was born of first-cousin parents, and her two younger brothers are healthy. The disease was diagnosed in her teens and progressed slowly afterwards. Mild foot dystonia, hyperreflexia and diurnal fluctuation of symptoms were observed, in addition to the parkinsonian tetrad. Her response to levodopa was remarkable, but dopa-induced dyskinesia occurred frequently. The clinical features of two patients in family 2 (patients II-1 and II-2) and of two other patients in unrelated families are similar to those of patient IV-1 in family 1, but the two patients in family 2 were born to unrelated parents. The age of onset was from 18 to 27 years in family 2. Patient II-2 had an autopsy and a small piece of his brain tissue was used for RT-PCR analysis.

Screening of BAC clones. Initially, two BAC clones (KB761D4 and KB430C4) were screened from a Keio BAC library consisting of 96,000 BAC clones by PCR using amplimers for D6S305 (ref. 6). An additional 24 BAC clones were isolated by hybridization with a cDNA containing an ORF against 95,232 clones using 31 high-density replica filters⁶. One more BAC clone was obtained from the

Keio BAC library by PCR with amplimers for exon 1, and two more BAC clones were purchased from Genome Systems by ordering PCR screening for exon 4. **Exon trapping and cDNA screening.** Exon trapping was performed using BAC DNAs of KB761D4 and KB430C4 and an exon-amplification kit (GIBCO/BRL). Human cDNA libraries (Clontech) of fetal brain and skeletal muscle were screened with a putative exon (J-17) and several cDNAs as probes. Positive clones were selected, and cDNA inserts were amplified by vector-specific primers (F10 inner, 5'-AGCCTGGTTAAGTCCAAGCTG-3'; R10 inner, 5'-GAAGGTCCCATTTTTCGTTTTC-3'). The amplified fragments were sequenced directly by the primer walking method. Cycle sequencing was performed using the primers and an ABI PRISM labelling kit (Perkin-Elmer) on the ABI model 377 DNA sequencer (Applied Biosystems).

Direct BAC sequencing. Oligonucleotide primers were designed according to the cDNA sequence. Sequencing used oligonucleotide primers and BAC DNAs as templates for the ABI PRISM labelling kit and the ABI model 377 DNA sequencer. **PCR amplification of exons and DNA sequencing.** DNA was isolated from peripheral blood leukocytes²⁷. Fourteen sets of oligonucleotide primers were designed according to the exon and flanking intron sequences (primer sequences and the sizes of the expected PCR products of 12 exons are available as Supplementary Information). PCR amplification was carried out in 10-μl

reactions, each of which contained 100 ng DNA, PCR buffer (50 mM Tris-HCl (pH 9.2 at 25 °C), 14 mM (NH₄)₂SO₄, 1.75 mM MgCl₂), 350 µM of each dNTP, 0.5 µM of each primer and 0.35 U expand long *Taq* polymerase (Boehringer). PCR conditions were 35 cycles at 94 °C for 30 s, 50–53 °C for 30 s, 68 °C for 30 s–1 min. Cycle sequencing used the appropriate PCR primers and a commercial kit.

Southern and northern blot analysis. Genomic DNA was extracted from the blood leukocytes of the Family 1 members and from FLEB 14-14 cells. Three micrograms of each genomic DNA were digested with *Eco*RI, electrophoresed and blotted onto nylon membrane. ³²P-labelling of cDNA clones was done by random primer extension. Hybridization was done as described²⁷. Multiple-tissue northern blots were purchased (Clontech) and hybridized as directed.

RT-PCR. Total cellular RNA was isolated from the brain tissue of patient II-2 in family 2 according to the acid guanidinium thiocyanate-phenol-chloroform procedure²⁷. Reverse transcription of 1 mg total RNA was primed at 50 °C for 30 min using a Titan one-tube RT-PCR system kit (Boehringer Mannheim), and the reaction mixture was directly used for PCR with forward primer (nucleotides 351–371) 5'-GGAGGCGACGACCCCA GAAAC-3' and reverse primer (nucleotides 963–983) 5'-GGGACAGCCAGCCACACAAGG-3'. PCR was done at 94 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min and repeated for 45 cycles.

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1. Parkinson, J. *An Essay on the Shaking Palsy* (Whittingham and Rowland, London, 1817).
2. Yamamura, Y., Arihiro, K., Kohriyama, T. & Nakamura, S. Early-onset parkinsonism with diurnal fluctuation-clinical and pathological studies. *Clin. Neurol. (Tokyo)* **33**, 491–496 (1993).
3. Ishikawa, A. & Tsuji, S. Clinical analysis of 17 patients in 12 Japanese families with autosomal recessive type juvenile parkinsonism. *Neurology* **47**, 160–166 (1996).
4. Matsumine, H. *et al.* Localization of a gene for autosomal recessive form of juvenile parkinsonism (AR-JP) to chromosome 6q25.2-27. *Am. J. Hum. Genet.* **60**, 588–596 (1997).
5. Matsumine, H. *et al.* Evidence for a microdeletion of D6S305 in a family of autosomal recessive juvenile parkinsonism (AR-JP). *Genomics* (in the press).
6. Asakawa, S. *et al.* Human BAC library: Construction and rapid screening. *Gene* **191**, 69–79 (1997).
7. Finley, D. & Chau, V. Ubiquitination. *Annu. Rev. Biol.* **7**, 25–69 (1991).
8. Saurin, A. J., Borden, K. L. B., Boddy, M. N. & Freemont, P. S. Does this have a familiar RING? *Trends Biochem. Sci.* **21**, 208–214 (1996).
9. Asland, R., Gibson, T. J. & Stewart, A. F. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.* **20**, 56–59 (1996).
10. Takahashi, H. *et al.* Familial juvenile parkinsonism: clinical and pathologic study in a family. *Neurology* **44**, 437–441 (1994).
11. Calne, D. B. & Langston, J. W. Aetiology of Parkinson's disease. *Lancet* **ii**, 1457–1459 (1983).
12. Jenner, P., Schapira, A. H. V. & Marsden, C. D. New insights into the cause of Parkinson's disease. *Neurology* **42**, 2241–2250 (1992).
13. Schapira, A. H. V. *et al.* Mitochondrial complex I deficiency in Parkinson's disease. *J. Neurochem.* **54**, 823–827 (1990).
14. Mizuno, Y. *et al.* Role of mitochondria in the etiology and pathogenesis of Parkinson's disease. *Biochim. Biophys. Acta* **1271**, 265–274 (1995).
15. Spillantini, M. G. *et al.* α -Synuclein in Lewy bodies. *Nature* **388**, 839–840 (1997).
16. Polymeropoulos, M. H. *et al.* Mutation in α -synuclein gene identified in families with Parkinson's disease. *Science* **276**, 2045–2047 (1997).
17. Mori, H., Kondo, J. & Ihara, Y. Ubiquitin is a component of paired helical filaments in Alzheimer's disease. *Science* **235**, 1641–1644 (1987).
18. Morishima-Kawashima, M. *et al.* Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. *Neuron* **10**, 1151–1160 (1993).
19. Gregori, L., Fuchs, C., Figueiredo-Pereira, M. E., Nostrand, W. E. V. & Goldgaber, D. Amyloid β -protein inhibits ubiquitin-dependent protein degradation *in vitro*. *J. Biol. Chem.* **270**, 19702–19708 (1995).
20. Love, S., Saitoh, T., Quijada, S., Cole, G. M. & Terry, R. D. Alz-50, ubiquitin and Tau immunoreactivity of neurofibrillary tangles, Pick bodies and Lewy bodies. *J. Neuropathol. Exp. Neurol.* **47**, 393–405 (1988).
21. Galloway, P. G., Mulvihill, O. & Perry, G. Filaments of Lewy bodies contain insoluble cytoskeletal elements. *Am. J. Pathol.* **140**, 809–822 (1992).
22. Iwatsubo, T. *et al.* Purification and characterization of Lewy bodies from the brains of patients with diffuse Lewy body disease. *Am. J. Pathol.* **148**, 1517–1529 (1996).
23. Ciechanover, A. The ubiquitin-proteasome proteolytic pathway. *Cell* **79**, 13–21 (1994).
24. Hochstrasser, M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr. Opin. Cell Biol.* **7**, 215–223 (1995).
25. Gregori, L., Poesch, M. S., Cousins, G. & Chau, V. A uniform isopeptide-linked multiubiquitin chain is sufficient to target substrate for degradation in ubiquitin-mediated proteolysis. *J. Biol. Chem.* **265**, 8354–8357 (1990).
26. Finley, D., Bartel, B. & Varshavsky, A. The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* **338**, 394–401 (1989).
27. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

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Correspondence and requests for materials should be addressed to N.S. (e-mail: shimizu@dmf.med.keio.ac.jp). The nucleotide sequences of cDNA for Parkin and intron-exon boundaries have been deposited in the DDBJ database (accession number AB009973).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells

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The dramatic increase in sequence information in the form of expressed sequence tags (ESTs)¹ and genomic sequence has created a 'gene function gap', with the identification of new genes far outpacing the rate at which their function can be identified. The ability to create mutations in embryonic stem (ES) cells on a large scale by tagged random mutagenesis provides a powerful approach for determining gene function in a mammalian system; this approach is well established in lower organisms^{2,3}. Here we describe a high-throughput mutagenesis method based on gene trapping that allows the automated identification of sequence tags from the mutated genes. This method traps and mutates genes regardless of their expression status in ES cells. To facilitate the study of gene function on a large scale, we are using these techniques to create a library of ES cells called Omnibank, from which sequence-tagged mutations in 2,000 genes are described.

Gene trapping is a method of random insertional mutagenesis that uses a fragment of DNA coding for a reporter or selectable marker gene as a mutagen^{4,5}. The marker can only be expressed if it inserts within an intron, an exon or a promoter of a transcriptionally active gene. Although such a strategy allows for the selection of insertion events in genes and tags the genes for subsequent cloning, the most notable limitation of previous trapping vectors is that only active genes are targets, restricting the set of genes that can be mutated in any particular cell type. To date, most trap designs have not been optimized to allow automation of the trapped gene identification^{6–9}, and the sequence tags often correspond to non-transcribed or 5' untranslated regions. Such sequence information is underrepresented in genomic, complementary DNA and EST databases and is less conserved between species, making it difficult to match the mutated gene to a known gene. To overcome these limitations, we developed gene trap vectors (Fig. 1a; VICTR3 and VICTR20) based on two functional units: (1) a sequence acquisition component consisting of a promoter (from the mouse gene encoding phosphoglycerate kinase-1) active in ES cells fused to the puromycin *N*-acetyltransferase gene coding sequence that lacks a polyadenylation sequence but is followed by a synthetic consensus splice donor sequence (PGKpuroSD); and (2) a mutagenic component that consists of a splice acceptor sequence fused to a selectable, colorimetric marker gene followed by a polyadenylation sequence (SA β geobpA or SAIRE β geobpA).

We anticipated that puromycin resistance could be achieved only by splicing into exons downstream of a trapped gene and addition of a polyadenylation sequence (Fig. 1b). In this design, a gene need not be transcribed to be trapped and the sequence of the trapped gene can be obtained by rapid amplification of cDNA ends (3' RACE), a simple, automatable procedure. The resulting sequence of the fusion transcript is informative in database searches because it is likely to contain coding regions. To determine whether PGKpuroSD could trap a gene as predicted, it was targeted by homologous recombination to the second intron of the *Hprt* gene. Sequencing of the 3' RACE products from puromycin-resistant colonies demonstrated that these colonies were the result of successful targeting events (targeting of *Hprt* was confirmed by Southern blot analysis; data not shown).

Our gene trap strategy relies on the effective mutagenesis of the

trapped genes. Previous studies of gene trap vectors using SA β geobpA have shown that it can disrupt endogenous messages and create null alleles^{6,7,10,11}. We tested both VICTR20 and PGKpuroSD independently for their ability to mutagenize the *Hprt* locus. Gene targeting of VICTR20 to the second intron of *Hprt* resulted in colonies resistant to 6-thioguanine, indicating disruption of wild-type *Hprt*. In contrast, targeting PGKpuroSD alone to the same position in *Hprt* did not yield colonies resistant to 6-thioguanine. Targeting for all constructs at the *Hprt* locus was confirmed by Southern analysis (Fig. 2). Although these data do not guarantee that VICTR20 will be mutagenic at all loci, they support previous data that most gene trap events containing SA β geobpA in a retrovirus will result in a null allele.

Having established the basic functionality of our gene trap constructs, we expanded the application to a large-scale mutagenesis program. ES cells were electroporated with a PGKpuroSD plasmid or infected with recombinant VICTR3 or VICTR20 retrovirus produced from a MoMLV-based packaging cell line. After transduction of the gene trap vectors, puromycin selection produced clonal lines. Lysis of cells from each clone provided RNA for 3' RACE. Direct sequencing of 3' RACE products yielded high-quality sequence 80–700 nucleotides long from 75% of the clones when the process was performed manually, and from 60% when performed robotically. The sequence tag from each clone, termed an Omnibank sequence tag (OST), was entered into a relational database for analysis and archiving. The first 3,000 OSTs were compared to GenBank (version 100.0) and SwissProt (version 34) databases using the BLASTN or BLASTX algorithm¹², respectively, to search for sequence similarities. Compiling all matches that result in a BLASTN probability score of 10^{-30} or less, we found that 18% of OSTs match known genes (see Supplementary Information), 10% match human and rodent ESTs, 10% match repeats such as B1, B2 or transposable elements, and 61% do not match any sequences in the databases.

To demonstrate that OSTs can identify the gene that is trapped

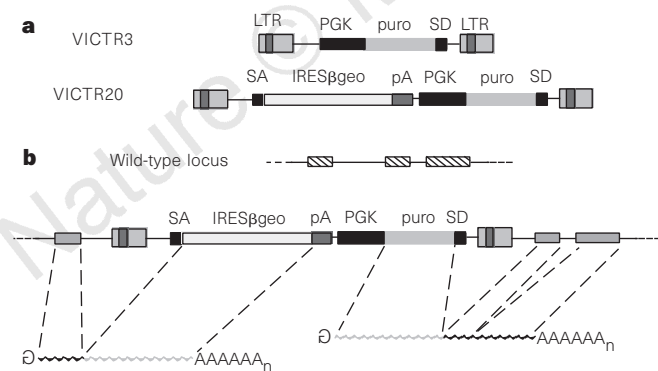


Figure 1 Vector design for 3' trapping. **a**, The retroviral constructs VICTR3 (for viral construct for trapping) and VICTR20 are drawn schematically. The splice donor of PGKpuroSD in VICTR3 and VICTR20 is preceded by stop codons in all three reading frames to prevent expression of fusion transcripts as well as unique sequences for nested 3' RACE and direct sequencing. The splice donor was added to puromycin using a PCR primer containing a unique sequence for 3' RACE, the sequence of the OBS sequencing primer, and a consensus splice donor sequence (5'-CCG CTC GAG ACT TAC CTG ACT GGC CGT CGT TTT AA GAC GAG CTC CCT AGC TAG TCA GGC ACC GGG CTT-3'). LTR, long terminal repeat; PGK, phosphoglycerate kinase-1 promoter; puro, puromycin *N*-acetyltransferase gene; SD, splice donor sequence; SA, splice acceptor sequence; IRES, internal ribosome entry site; β geo, galactosidase/neomycin phosphotransferase fusion gene; pA, polyadenylation sequence. **b**, A representation of a VICTR20 retroviral insertion into a three-exon gene. The resulting integration would produce two fusion transcripts, one containing puro sequences at the 5' end and sequences from the trapped gene at the 3' end, and the other containing sequences of the trapped gene at the 5' end and β geo sequences at the 3' end.

and determine the position of retroviral integration, we carried out Southern blot analysis on DNA isolated from a gene trap event at the Bruton's tyrosine kinase (*btk*) locus¹³. The *btk* OST sequence began with exon 2, suggesting that the insertion occurred in the first intron of the *btk* gene. Southern blot analysis using several different restriction enzymes confirmed the location of the retroviral insertion in the first intron of the gene (Fig. 3). Comparing OST sequences to cDNA sequences for 108 known genes revealed that 44% of the viral integration events were near the 5' ends of genes (within the first 350 nucleotides of the 5' end of the encoded cDNA), 44% were in the middle of genes, and 12% were near the 3' end of genes (within 350 nucleotides of the 3' end of the encoded cDNA). Our data suggest that, although there may be some bias towards 5' integrations, VICTR trap events integrate throughout genes.

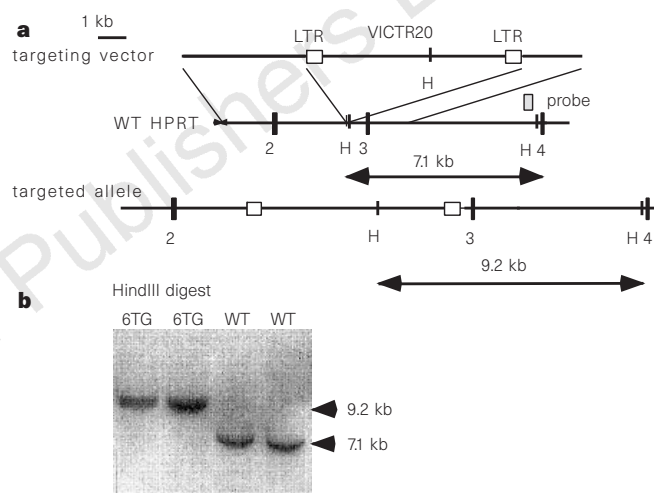


Figure 2 Targeting of VICTR20 to the second intron of the *Hprt* locus. *Hprt* targeting experiments were carried out as previously described²⁰. PGKpuroSD and VICTR20 were placed into the *Eco*RI site of pRIV6.9I. **a**, The targeting vector, wild-type *Hprt* allele and targeted allele are shown. Open rectangles denote the retroviral LTRs and the black numbered rectangles denote exons 2, 3 and 4 of *Hprt*. (H) *Hind*III. **b**, Southern blot of the targeted 6-thioguanine resistant (6-TG) clones versus non-targeted wild-type (WT) clones. The blot was probed with sequences 5' of exon 4.

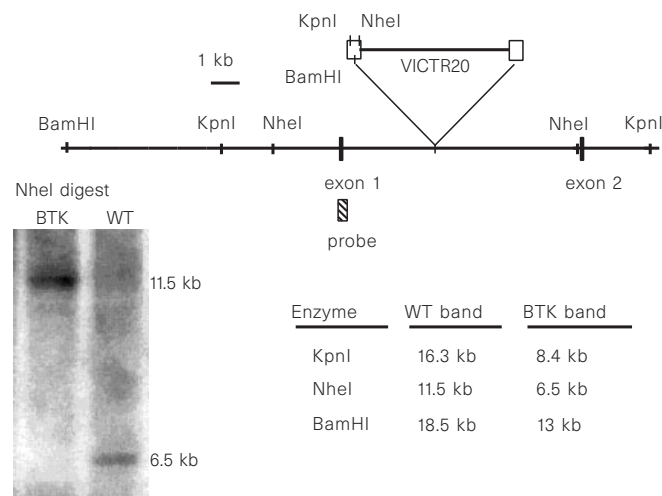


Figure 3 VICTR20 has integrated into the first intron of the *btk* gene. The diagram shows the position of retroviral integration into the *btk* locus. The Southern blot shows the *Nhe*I restriction-fragment length polymorphism produced by VICTR20 integration and the table shows the fragment sizes observed after digestion of DNA from wild type versus the *btk* gene trap ES cells.

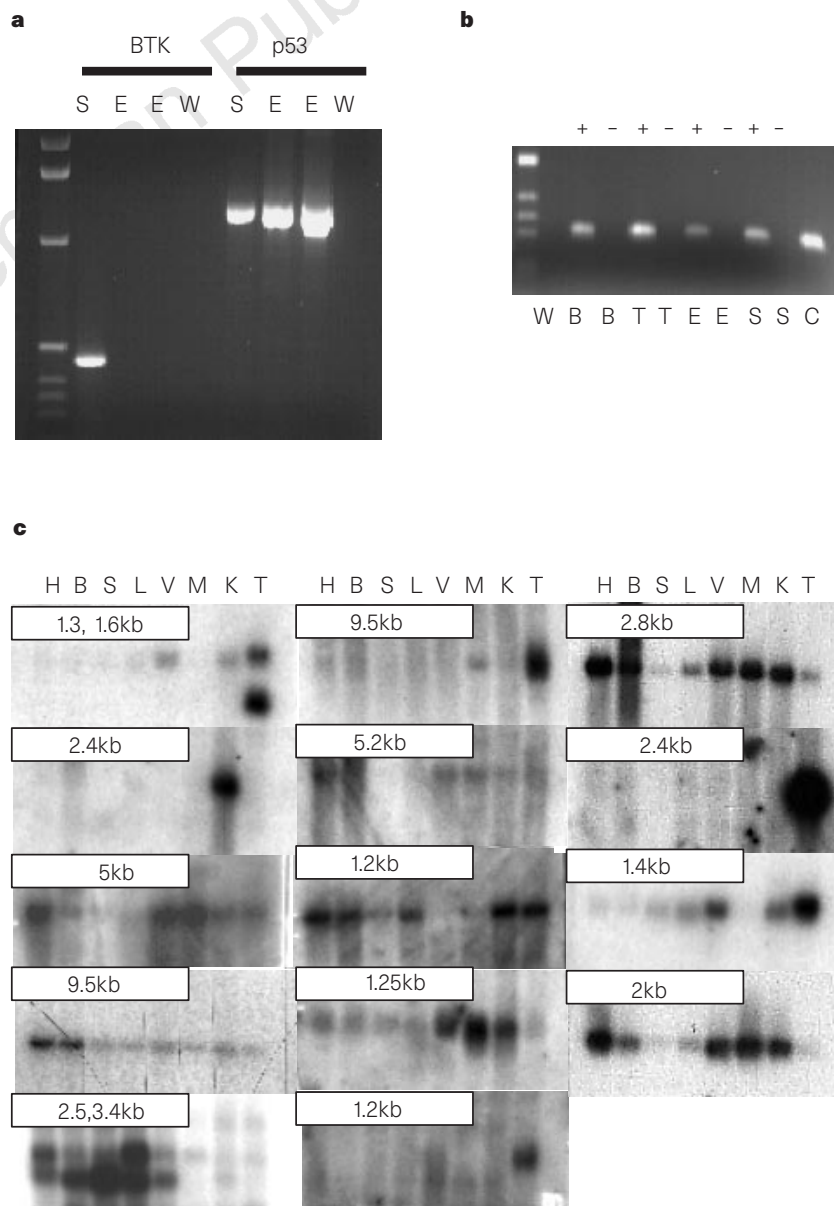
We predicted that the gene trap design should not rely on expression of the target gene. The current set of OSTs contains many genes that are known to have restricted patterns of expression and were thus unlikely to be expressed in ES cells. These include the *btk* gene known to be expressed in B lymphocytes¹⁴. We detected *btk* expression in the spleen by an RT-PCR (polymerase chain reaction after reverse transcription of RNA) assay but were unable to detect expression in ES cells (Fig. 4a). Similarly, several OSTs with unique sequences could not be detected in ES cells (data not shown), confirming the ability of the gene trap vectors to trap genes that are not expressed in ES cells.

Sequence analysis of over 3,000 OSTs has demonstrated a high stringency for splicing to generate drug-resistant colonies, as only 10 of the OSTs contain vector sequence that follows the synthetic splice donor. Nonetheless, the large fraction of new OSTs (61%) made it important to demonstrate that they represented transcribed sequences. We chose 37 of the novel or unknown OSTs at random (17 produced by electroporation and 20 produced by retroviral infection) for expression analysis using RT-PCR (Fig. 4b). Of the 37 OSTs, 34 were expressed in brain, testis, ES cells or day-10.5 embryos. To demonstrate further that the OSTs correspond to exonic sequence and not unspliced nuclear RNAs, we probed

northern blots of poly(A)⁺ RNA from multiple tissues with 16 unknown OSTs (Fig. 4c). Of 16 of the OST probes, 14 hybridized to discrete messenger RNAs at high stringency, suggesting that the unknown OST sequences are present in mRNA and represent exonic sequences of genes. Because the 3' RACE products tend to be longer than the initial OST obtained, additional sequence was obtained from the 3' RACE products of 290 unknown OSTs. This provided 70–700 base pairs (an average of 263 bp) of additional sequence per OST, and BLASTN searches revealed that 27% of these expanded OSTs matched sequences in GenBank (34 ESTs, 43 known genes). These results provide further support that VICTR gene trap vectors are trapping transcribed sequences. The expanded OST sequences average about 500 bases in length, yet 74% remain unknown and may represent newly discovered genes.

Our expression-independent method of sequence acquisition is unlikely to suffer from the same biases inherent to EST acquisition methods that typically result in underrepresentation of genes that are expressed at low levels, transiently or only in limited cell types. Gene trap methods, however, are not without selective pressures affected by vector design, delivery, target gene size and gene accessibility, which are likely to produce some level of bias in the relative representation of trapped genes. It is encouraging that

Figure 4 Expression of 'unknowns'. **a**, First-strand cDNA was synthesized using RNA from spleen (S), two ES cell RNA samples (E) or water (W). PCR was carried out on the first strand cDNA using primers specific for *btk* or p53 transcripts. The integrity of the ES cell RNA and reverse transcription reactions was confirmed using primers for p53, a gene known to be expressed in ES cells. **b**, First-strand cDNA was synthesized using RNA isolated from brain (B), testis (T), day 10.5 embryos (E) and ES cells (S). First-strand cDNA reactions were carried out in the presence (+) or absence (–) of reverse transcriptase and a water (W) and positive control (C) were included. PCR was carried out on first-strand cDNA using primers specific for 37 different unknown OSTs. **c**, Ten unknown OSTs were used as probes on multitissue northern blots made with poly(A)⁺ RNA. The blots were washed at high stringency. H, heart; B, brain; S, spleen; L, lung; V, liver; M, skeletal muscle; K, kidney; T, testis.



several reports suggest that most of the genome is accessible to retroviral integration^{15,16} with a subset of the integration events landing in 'hotspots', and with the remainder representing random integrations¹⁷. Our data support this model because most genes in Omnibank are represented by single gene trap events. Additionally, this percentage of single hit genes has remained stable as the number of OSTs has increased. To provide support that VICTR20 has a large target size and is mutagenic, a large-scale infection of ES cells was carried out, followed by selection for mutations in *Hprt* using 6-thioguanine. One 6-thioguanine-resistant colony was identified in 80,000 gene trap events and Southern blot analysis and RT-PCR confirmed it to be the result of an integration in the *Hprt* locus (data not shown). Although the gene target size for VICTR20 will become apparent as the OST numbers grow, our data suggest that the number of potential target genes is large.

We have automated the processing of mutagenized ES cells in the 96-well plate format resulting in the addition of at least 500 OSTs to the Omnibank library each week. This library represents a new type of functional genomics resource as it both contains sequence information of mutated genes and provides the ability to rapidly produce mouse mutants to analyse the function of large groups of genes. Future work will involve the production and analysis of mice from this resource and the further development of vectors and delivery systems that will maximize the number of unique gene trap events in Omnibank. □

Methods

Vectors and cell culture. PGKpuroSD, SAβgeoPGKpuroSD and SAIRESDβgeoPGKpuroSD were all placed into the *Xho*I site of pGen[−] (ref. 18) in reverse orientation. VICTR3 and VICTR20 were packaged in the retroviral producer cell line GP+E86 as described¹⁸. Electroporation and retroviral infection were carried out as previously described and ES cells were selected for 10 days in 2.75 mg ml^{−1} puromycin (Sigma)^{18,19}. After expansion, two portions of each ES cell clone were stored in vapour-phase cryofreezers and one copy was processed to obtain sequence tags.

RNA isolation, 3' RACE and sequencing. RNA was isolated from ES cells grown on 35-mm plates using RNAzol (Tel-Test). First-strand cDNA synthesis and 3' RACE PCR was carried out as previously described⁷ using Qt primer to prime cDNA synthesis and two rounds of PCR using primers Qo and P₀ (5'-AAG CCC GGT GCC TGA CTA GCT AG-3'). PCR products were purified using Sephadex G-50 (DNA grade, Pharmacia) and sequenced with dye terminator cycle sequencing reactions and AmpliTaq FS DNA polymerase (Perkin Elmer). Reactions were carried out using 7 pmol of primer OBS (5'-CTG TAA AAC GAC GGC CAG TC-3') and approximately 30–120 ng of 3' RACE product. Reactions were run on an ABI Prism 377 with an XL upgrade according to the manufacturer's instructions.

RT-PCR and northern blotting. RT-PCR was carried out using 40 cycles and the reaction conditions described above for 3' RACE. Northern blots were prepared using Clontech multitissue blots and were washed at a high stringency of 65 °C in 0.2 × SSC, 0.1% SDS for 1 h.

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- Adams, M. D. *et al.* Complementary DNA sequencing: "Expressed sequence tags" and the Human Genome Project. *Science* **252**, 1651–1656 (1991).
- Plasterk, R. H. Reverse genetics of *Caenorhabditis elegans*. *Bioessays* **14**, 629–633 (1992).
- Spradling, A. C. *et al.* Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl Acad. Sci. USA* **92**, 10824–10830 (1995).
- Gossler, A. & Zachgo, J. in *Gene Targeting: A Practical Approach* (ed. Joyner, A. L.) 181–227 (Oxford Univ. Press, New York, 1993).
- Friedrich, G. & Soriano, P. Insertional mutagenesis by retroviruses and promoter traps in embryonic stem cells. *Meth. Enzymol.* **225**, 681–701 (1993).
- Chen, Z., Friedrich, G. A. & Soriano, P. Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. *Genes Dev.* **8**, 2293–2301 (1994).
- Zambrowicz, B. P. *et al.* Disruption of overlapping transcripts in the ROSA βgeo 26 gene trap strain leads to widespread expression of βgalactosidase in mouse embryos and hematopoietic cells. *Proc. Natl Acad. Sci. USA* **94**, 3789–3794 (1997).
- Townley, D. J., Avery, B. J., Rosen, B. & Skarnes, W. C. Rapid sequence analysis of gene trap integrations to generate a resource of insertional mutations in mice. *Genome Res.* **7**, 293–298 (1997).
- Hicks, G. G. *et al.* Functional genomics in mice by tagged sequence mutagenesis. *Nature Genet.* **16**, 338–344 (1997).
- Friedrich, G. & Soriano, P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513–1523 (1991).
- Skarnes, W., Moss, J., Hurlbert, S. & Beddington, R. Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc. Natl Acad. Sci. USA* **92**, 6592–6596 (1995).

- Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
- Oeltjen, J. C. *et al.* Large-scale comparative sequence analysis of the human murine Bruton's tyrosine kinase loci reveals conserved regulatory domains. *Genome Res.* **7**, 315–329 (1997).
- de Weers, M. *et al.* The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* **23**, 3109–3114 (1993).
- Chang, W., Hubbard, C., Friedel, C. & Ruley, E. Enrichment of insertional mutants following retrovirus gene trap selection. *Virology* **193**, 737–747 (1993).
- Withers-Ward, E. S., Kitamura, Y., Barnes, J. P. & Coffin, J. M. Distribution of targets for avian retrovirus DNA integration *in vivo*. *Genes Dev.* **8**, 1473–1487 (1994).
- Craigie, R. Hotspots and warm spots: integration specificity of retroelements. *Trends Genet.* **8**, 187–190 (1992).
- Soriano, P., Friedrich, G. & Lawinger, P. Promoter interaction in retrovirus vectors introduced into fibroblasts and embryonic stem cells. *J. Virol.* **65**, 2314–2319 (1991).
- Zhu, B., Bogue, M. A., Lim, D.-S., Hasty, P. & Roth, D. B. Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell* **86**, 379–389 (1996).
- Hasty, P., Rivera-Perez, J., Chang, C. & Bradley, A. Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol. Cell. Biol.* **11**, 4509–4517 (1991).

Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Correspondence and requests for materials should be addressed to B.P.Z. (e-mail: materials@lexgen.com). Sequences of clones given in Supplementary Information have been deposited in GenBank, accession numbers AF046239–AF046781. Lexicon will distribute the ES cell clones described here to requesting investigators for non-commercial research.

Role of Rel/NF-κB transcription factors during the outgrowth of the vertebrate limb

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The development of the vertebrate limb serves as an amenable system for studying signaling pathways that lead to tissue patterning and proliferation¹. Limbs originate as a consequence of a differential growth of cells from the lateral plate mesoderm at specific axial levels². At the tip of the limb primordia the progress zone, a proliferating group of mesenchymal cells, induces the overlying ectoderm to differentiate into a specialized structure termed the apical ectodermal ridge. Subsequent limb outgrowth requires reciprocal signalling between the ridge and the progress zone^{3–6}. The Rel/NF-κB family of transcription factors is induced in response to several signals that lead to cell growth, differentiation, inflammatory responses, apoptosis and neoplastic transformation⁷. In unstimulated cells, NF-κB is associated in the cytoplasm with an inhibitory protein, I-κB. In response to an external signal, I-κB is phosphorylated, ubiquitinated and degraded, releasing NF-κB to enter the nucleus and activate transcription⁷. Here we show that Rel/NF-κB genes are expressed in the progress zone of the developing chick limb bud. When the activity of Rel/NF-κB proteins is blocked by infection with viral vectors that produce transdominant-negative I-κBα proteins, limb outgrowth is arrested. Our results indicate that Rel/NF-κB transcription factors play a role in vertebrate limb development.

Rel/NF-κB transcripts are first detected in the chick presumptive limb bud mesoderm at stage 15 HH⁸, coincident with the first sign of limb budding (Fig. 1a). As limb outgrowth proceeds, transcripts

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become confined to the most distal mesodermal cells (Fig. 1b–d). These cells, together with the apical ectodermal ridge (AER), are responsible for maintaining the outgrowth of the limb. The expression of Rel/NF- κ B at the distal end of the limb bud is maintained until the latest stage examined (stage 29; Fig. 1e). The expression pattern of Rel/NF- κ B in the distal most mesoderm overlaps with the pattern of expression of *Msx-1* (ref. 9), *Lhx-2* (J.C.I.B., unpublished

data) and *Twist*¹⁰, genes known to be involved in limb outgrowth (Fig. 1f–h).

To determine whether continued Rel/NF- κ B expression might depend on signals from the AER, we surgically removed the ridge at stages 18–20 HH and subsequently observed a rapid loss of Rel/NF- κ B expression (Fig. 1i, j). After 4 h, Rel/NF- κ B transcripts were partially downregulated at the most distal tip, and transcripts were

Figure 1 Expression of Rel/NF- κ B and other proteins involved in chick limb development. **a–e**, Spatio-temporal pattern of expression of Rel/NF- κ B during limb bud development. **a**, Whole-mount *in situ* hybridization showing Rel/NF- κ B transcripts at stage (St.) 15 in the presumptive limb mesenchyme. **b–d**, At stages 17–25, Rel/NF- κ B expression becomes restricted to the most distal limb bud mesoderm. **e**, In addition to being located distally, at stage 29, Rel/NF- κ B transcripts appear faintly in the presumptive interdigital areas. White arrows indicate Rel/NF- κ B expression. Rel/NF- κ B transcripts were not detected in the limb bud ectoderm. **f, g**, Restricted expression of *Msx-1* and *Lhx-2* transcripts in the mesoderm. There is an overlapping domain of expression of these genes and of Rel/NF- κ B. **h**, *Twist* transcripts are detected all over the mesenchyme, with a higher density towards the distal tip of the limb bud. **k**, Regulation of Rel/NF- κ B expression by FGF. Surgical removal of the AER results in loss of Rel/NF- κ B expression. Embryos are shown 4 h (**i**) and 24 h (**j**) after AER removal. When a bead soaked in FGF protein is implanted after AER removal, Rel/NF- κ B expression is restored after 24 hours (**k**). Black arrows indicate the manipulated limb buds.

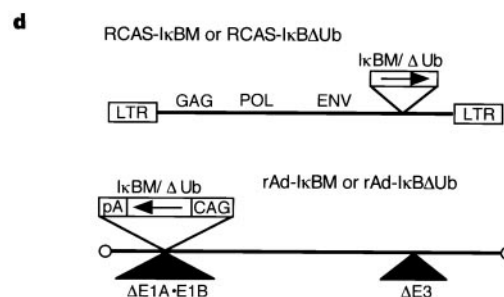
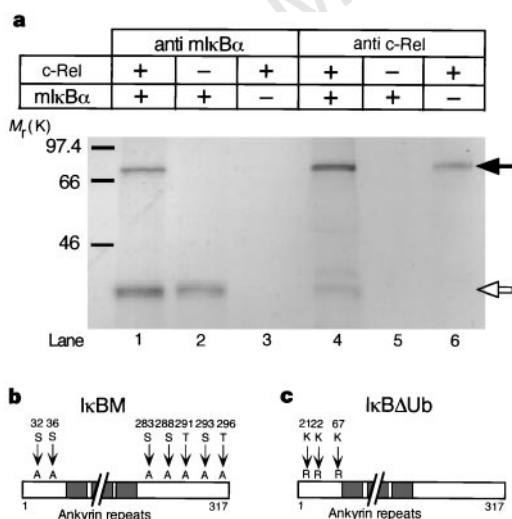
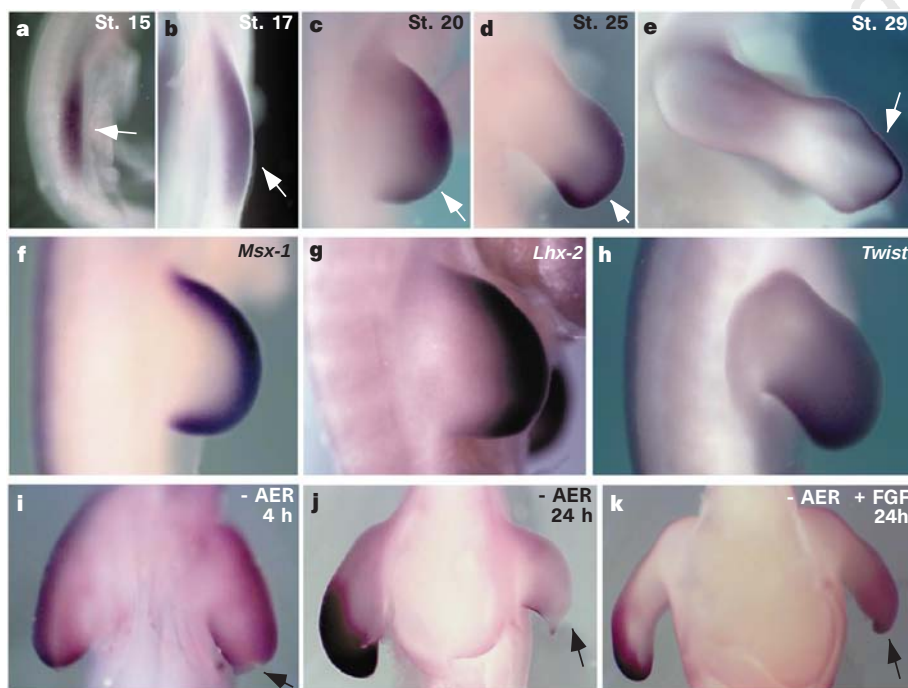


Figure 2 Characterization of transdominant-negative mutants of mouse IκBα. **a**, Association between chicken Rel and mouse IκBα. The cDNAs encoding mouse IκBα and chicken c-Rel were transcribed and translated *in vitro* and immunoprecipitated with appropriate antibodies. The filled arrow indicates the migration of chicken c-Rel, and the open arrow denotes migration of mIκBα. **b, c**, Schematic representations of the mouse IκBα transdominant-negative mutants IκBM (which has mutated phosphorylation sites) and IκBΔUb (which has mutated lysine residues). **d**, RCAS (replication-competent avian retrovirus) vectors (top) and recombinant adenoviral (rAd) vectors (bottom).

Arrows indicate the orientation of the transcription. LTR, long terminal repeat; Gag, Env and Pol are HIV proteins. **e**, Stability of IκBα mutants (IκBM and IκBΔUb). 10⁷ HeLa cells were infected with adenoviral vectors containing wild-type (WT) IκBα (lanes 4–6), IκBM (lanes 7–9) and IκBΔUb (lanes 10–12) at a multiplicity of infection (MOI) of 3. 10 ng TNF- α per ml were added to cells at 24 h after infection. IκBα proteins were analysed using western blotting. The open arrow indicates the position of IκBα (human); the solid arrows indicate transduced murine IκB and phosphorylated IκB.

undetectable by 24 h after AER removal. The AER signal responsible for maintaining cell proliferation and gene expression in the progress zone can be replaced with members of the fibroblast growth factor (FGF) family^{11–16}. Use of an FGF-soaked bead after ridge removal restored apparently normal expression of Rel/NF- κ B (Fig. 1k). These data support the concept of a feedback loop between cells in the AER and cells expressing Rel/NF- κ B transcripts in the underlying mesoderm. Our results establish, for the first time, a relationship between the Rel/NF- κ B and FGF gene families during vertebrate development.

We constructed two types of transdominant-negative mutants of mouse I- κ B α (mI- κ B α) to block NF- κ B activity (Fig. 2b). In one case, the inducible phosphorylation sites (Ser 32 and Ser 36) and constitutive phosphorylation site at its carboxy terminus were substituted with alanine residues (I- κ BM mutant)¹⁷. The other mutant was generated by substituting three lysine residues presumed to be the ubiquitination sites on mI- κ B α (I- κ B Δ Ub). As the transdominant mutants of mI- κ B α are of mouse origin, we wanted to ensure that mI- κ B α can associate with avian Rel/NF- κ B protein. Figure 2a shows the association between *in vitro* translated mI- κ B α and chicken Rel/NF- κ B by coimmunoprecipitation (lanes 1, 4). These results concur with the association of chicken I- κ B α with mouse Rel/NF- κ B¹⁸. To show that mutant I- κ B α is not degraded upon stimulation with tumour-necrosis factor- α (TNF- α), we infected HeLa cells with adenoviral vectors containing wild-type I- κ B α , I- κ BM or I- κ B Δ Ub. Figure 2e shows that, in uninfected cells (lanes 1–3) and cells infected with wild-type I- κ B α (lanes 4–6), I- κ B α is rapidly degraded upon stimulation with TNF- α . In contrast, I- κ B α is stable in cells transduced with I- κ BM (lanes 7–9) or I- κ B Δ Ub (lanes 10–12) (see also Supplementary Information).

Limb buds infected with both transdominant-negative retroviral and adenoviral constructs were reduced in size and failed to develop a defined AER (Fig. 3b, c, and data not shown). When incubated with viral constructs for up to 10 days, severely truncated limbs with deformed and/or absent zeugopodal elements (most commonly absence of the radius) and missing digits were observed (Fig. 3f, g). After limb budding, continued limb outgrowth depends on correct AER formation. If the AER is disrupted or removed, cell proliferation is affected and limb outgrowth is arrested^{3,5,6}. The reduced limb buds seen after viral infection with the transdominant-negative I- κ B constructs closely resemble the limb buds obtained after AER removal (Fig. 3d, h). Our results indicate that downregulation of Rel/NF- κ B activity may perturb maintenance of AER function and hence limb outgrowth.

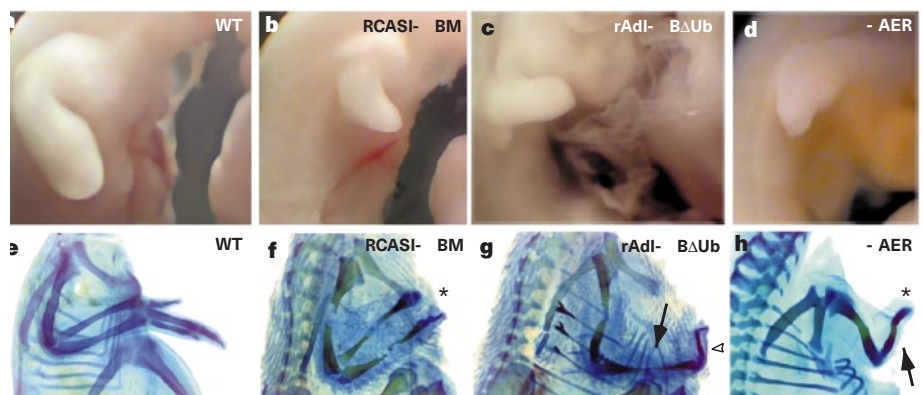
The phenotypes obtained using the adenoviral and retroviral vectors were indistinguishable. As high-titre recombinant adenovirus can be readily obtained (10^{10} – 10^{11} plaque-forming units (PFU) per ml) and as recombinant adenovirus can be used to transduce postmitotic cells, the use of recombinant adenovirus will be valuable as a means of inducing ectopic gene expression in

embryos. The size of the insert that can be accommodated by the adenoviral vector is much larger (>7 kilobases (kb)) than the insert size for currently available retroviral vectors (limited to ~ 2 kb).

We further characterized the expression of several molecular markers in both limb bud ectodermal and mesodermal cells following viral infection. *Fgf-8* is a gene whose expression is specifically restricted to the AER and which is involved in limb initiation and in maintaining limb outgrowth^{15,16}. *In situ* hybridization indicates that expression of *Fgf-8* is indeed downregulated or absent in the infected limb buds (Fig. 4a, b). Attenuation of expression of *Fgf* genes in the AER leads to a loss of *Shh* expression in the posterior mesoderm, in turn abolishing the expression of *Fgf* genes in the AER^{13,14}. Consistent with this, the expression of *Shh*, a gene that regulates antero–posterior patterning and which is required for distal outgrowth^{13,14,19}, is downregulated after viral infection (Fig. 4e). Furthermore, levels of transcripts of *Msx-1* and *Lhx-2*, genes involved in limb outgrowth whose domains of expression overlap with Rel/NF- κ B messenger RNAs, are also downregulated (Fig. 4c, d). The downregulation of transcription of *Msx-1* could be a direct consequence of inhibiting Rel/NF- κ B activity, as a κ B-binding site has been found in the regulatory region of the human *Msx-1* gene²⁰. Thus, although blocking the translocation of Rel/NF- κ B proteins to the nucleus downregulates expression of both mesodermal and ectodermal markers, it is not known whether this downregulation occurs primarily in the mesoderm and indirectly in the AER, or, alternatively, whether downregulation of gene expression occurs initially in the AER and subsequently in the mesoderm.

A fly homologue of members of the Rel/NF- κ B family, Dorsal, is required for the establishment of dorso–ventral polarity during *Drosophila* embryogenesis. The function of Dorsal is mediated in part through the transcriptional activation of Twist, a basic helix–loop–helix protein²¹. To determine whether a similar mechanism takes place during the outgrowth of the vertebrate limb, we cloned a chick *Twist* gene and characterized its expression following viral infection with the transdominant-negative I- κ B mutants. *In situ* hybridization at 56 h after infection shows a marked decrease of *Twist* expression (normally present throughout the entire mesenchyme (Fig. 1h)) in the reduced limb bud (Fig. 4f). The downregulation of *Twist* expression and arrest in chick limb outgrowth is consistent with reports of mice lacking TWIST in which the AER is not formed¹⁰. In humans, TWIST mutations are associated with perturbations in limb outgrowth, which are probably due to an abnormal development of the AER^{22,23}. During *Drosophila* development, Twist is required for the transcription of genes encoding FGF receptors²⁴. In vertebrates, the role of FGFs in mediating both limb initiation and maintenance of limb outgrowth seems to be partly mediated through the activation of FGF receptors 1 and 2, as gain- and loss-of-function experiments with these receptors show an interference with normal limb outgrowth^{25,26}. These results indicate

Figure 3 Blocking Rel/NF- κ B signalling inhibits limb outgrowth. After viral infection at stages 10–16, chicken embryos were left to develop either for 72 h (a–c) or for 8 days (e–g). **a, e**, Wild-type limbs; **b, f**, RCAS-I- κ BM-infected limbs; **c, g**, rAd-I- κ B Δ Ub-infected limbs; **d, h**, limbs after AER removal. Arrows indicate the absence of intermediate bones (radius in **g** and **h**); the arrow-head indicates the absence of two digits (**g**); and asterisks indicate the absence of all digits (**f, h**). A similar phenotype was observed in RCAS-I- κ B Δ Ub- and rAd-I- κ BM-infected embryos (data not shown).



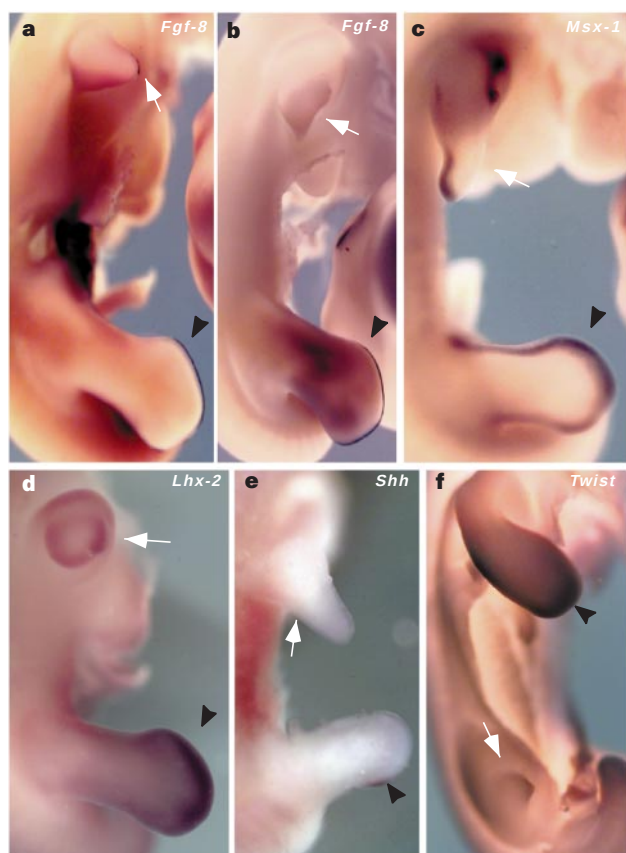


Figure 4 Blocking Rel/NF- κ B signalling alters gene expression in the developing limb. Sixty to seventy-two hours after viral infection, embryos were analysed by whole-mount *in situ* hybridization for the expression of mesodermal and ectodermal factors involved in limb outgrowth. Expression of *Fgf-8*, a marker for the AER, is reduced (**a**) or completely absent (**b**). Expression of progress-zone-specific markers *Msx-1* (**c**) and *Lhx-2* (**d**) is downregulated in the infected limbs. **e**, *Shh* expression, which is normally localized to the posterior region, was absent in the reduced limb. **f**, *Twist* expression is also downregulated in the infected limb. The white arrows indicate the altered gene expression patterns in infected limbs. The normal expression patterns of *Fgf-8*, *Msx-1*, *Lhx-2*, *Shh* and *Twist* are indicated by black arrowheads in non-infected limb buds.

the existence of a common signalling process mediating the activity of Rel/NF- κ B in both *Drosophila* and vertebrate development, and constitute a starting point in the unravelling of possible mechanisms through which Rel/NF- κ B proteins might regulate cell proliferation during limb development. However, ectopic expression of chicken c-Rel did not lead to significant morphological perturbations (data not shown). Thus, although our results indicate that Rel/NF- κ B proteins may control the proliferation and outgrowth of the vertebrate limb, some other factor(s) expressed in the limb primordia, such as *Msx-1*, *Lhx-2* or *Fgf-10*, probably cooperate(s) with Rel/NF- κ B in directing limb outgrowth. □

Methods

Embryo manipulations. Whole-mount *in situ* hybridizations, using digoxigenin probes for *Msx-1* (ref. 26), *Lhx-2* (our unpublished observations), *Twist* (Genbank database), *Fgf-8* (ref. 14) and *Shh*²⁸, were performed as described²⁹. The Rel digoxigenin antisense probe spans 1,850 base pairs of the mouse c-Rel complementary DNA. As this probe contains the highly conserved RHD amino-acid motif, it will probably recognize all the members of the Rel/NF- κ B family that are present at limb outgrowths. Similar results were observed when the 5' and 3' regions of chicken c-Rel were used as probes (see

Supplementary Information). AER removal, FGF application, viral infection (performed at stages 10–16), and embryo collection and analysis were done as described¹⁶. Perturbations in limb outgrowth were observed in ~10% of the infected embryos ($n = 585$). No alterations were seen following injection of viruses expressing the *Escherichia coli* β -galactosidase or the wild-type mI- κ B α genes. All recombinant adenoviral and replication-competent retroviral vectors were prepared as described^{16,30}.

Immunoprecipitations. c-Rel and mI- κ B α cDNAs were translated *in vitro* and immunoprecipitated with a chicken c-Rel (a gift from T. Gilmore) and the anti-MAD3/mI- κ B α antibodies according to manufacturer's instructions (Santa Cruz). The anti-MAD3/mI- κ B α antibodies were also used for western blotting.

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1. Tickle, C. & Eichele, G. Vertebrate limb development. *Annu. Rev. Cell Biol.* **10**, 121–152 (1994).
2. Searls, R. L. & Janners, M. Y. The initiation of limb bud outgrowth in the embryonic chick. *Dev. Biol.* **24**, 198–213 (1971).
3. Saunders, J. W. J. The proximo-distal sequence of the origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.* **108**, 363–404 (1948).
4. Kieny, M. Rôle inducteur du mésoderme dans la différenciation précoce du bourgeon de membre chez l'embryon de Poulet. *J. Embryol. Exp. Morphol.* **8**, 457–467 (1960).
5. Summerbell, D. A quantitative analysis of the effect of excision of the AER from the chick limb bud. *J. Embryol. Exp. Morphol.* **32**, 651–660 (1974).
6. Rowe, D. A. & Fallon, J. F. The proximodistal determination of skeletal parts in the developing chick leg. *J. Embryol. Exp. Morphol.* **68**, 1–7 (1982).
7. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. & Miyamoto, S. Rel/NF- κ B family: intimate tales of association and dissociation. *Genes Dev.* **9**, 2723–2735 (1995).
8. Hamburger, V. & Hamilton, H. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92 (1951).
9. Davidson, D. R., Crawley, A., Hill, R. E. & Tickle, C. Position-dependent expression of two related homeobox genes in developing vertebrate limbs. *Nature* **352**, 429–431 (1991).
10. Chen, Z. F. & Behringer, R. R. *Twist* is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9**, 686–699 (1995).
11. Niswander, L., Tickle, C., Vogel, A., Booth, I. & Martin, G. R. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579–587 (1993).
12. Fallon, J. F. et al. FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science* **264**, 104–107 (1994).
13. Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. & Tabin, C. *Sonic hedgehog* and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993–1003 (1994).
14. Niswander, L., Jeffrey, S., Martin, G. R. & Tickle, C. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609–612 (1994).
15. Crossley, P. H., Minowada, G., MacArthur, C. A. & Martin, G. R. Roles for FGF-8 in the induction, initiation and maintenance of chick limb development. *Cell* **84**, 127–136 (1996).
16. Vogel, A., Rodriguez, C. & Izpisua Belmonte, J. C. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737–1750 (1996).
17. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. & Verma, I. M. Suppression of TNF- α -induced apoptosis by NF- κ B. *Science* **274**, 787–789 (1996).
18. Inoue, J. et al. c-rel activates but v-rel suppresses transcription from kappa B sites. *Proc. Natl Acad. Sci. USA* **88**, 3715–3719 (1991).
19. Riddle, R. D., Johnson, R. L., Laufer, E. & Tabin, C. *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75**, 1401–1416 (1993).
20. Shen, R., Chen, Y., Huang, L., Vitale, E. & Solursh, M. Characterization of the human MSX-1 promoter and an enhancer responsible for retinoic acid induction. *Cell Mol. Biol. Res.* **40**, 297–312 (1994).
21. Jiang, J., Kosman, D., Ip, Y. T. & Levine, M. The dorsal morphogen gradient regulates the mesoderm determinant twist in early *Drosophila* embryos. *Genes Dev.* **5**, 1881–1891 (1991).
22. Howard, T. D. et al. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nature Genet.* **15**, 36–41 (1997).
23. el Ghouzzi, V. et al. Mutations of the TWIST gene in the Aethre-Chotzen syndrome. *Nature Genet.* **15**, 42–46 (1997).
24. Shishido, E., Higashijima, S., Emori, Y. & Saigo, K. Two FGF-receptor homologues of *Drosophila*: one is expressed in mesodermal primordium in early embryos. *Development* **117**, 751–761 (1993).
25. MacArthur, C. et al. FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. *Development* **121** (suppl.), 3603–3613 (1995).
26. Deng, C. et al. Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. *Dev. Biol.* **185**, 42–54 (1997).
27. Robert, B., Lyons, G., Simandl, B.-K., Kuroiwa, A. & Buckingham, M. The apical ectodermal ridge regulates Hox-7 and Hox-8 gene expression in developing limb buds. *Genes Dev.* **5**, 2363–2374 (1991).
28. Ogura, T. et al. Evidence that Shh cooperates with a retinoic acid inducible co-factor to establish ZPA-like activity. *Development* **122**, 537–542 (1996).
29. Izpisua Belmonte, J. C., De Robertis, E. M., Storey, K. G. & Stern, C. The homeobox gene *gooseoid* and the origin of organizer cells in the early chick blastoderm. *Cell* **74**, 645–659 (1993).
30. Miyake, S. et al. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl Acad. Sci. USA* **93**, 1320–1324 (1996).

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Inhibition of NF- κ B activity results in disruption of the apical ectodermal ridge and aberrant limb morphogenesis

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In *Drosophila*, the Dorsal protein establishes the embryonic dorso–ventral axis during development¹. Here we show that the vertebrate homologue of Dorsal, nuclear factor- κ B (NF- κ B), is vital for the formation of the proximo–distal organizer of the developing limb bud, the apical ectodermal ridge (AER). Transcription of the NF- κ B proto-oncogene *c-rel* is regulated, in part, during morphogenesis of the limb bud by AER-derived signals such as fibroblast growth factors. Interruption of NF- κ B activity using viral-mediated delivery of an inhibitor results in a highly dysmorphic AER, reduction in overall limb size, loss of distal elements and reversal in the direction of limb outgrowth. Furthermore, inhibition of NF- κ B activity in limb mesenchyme leads to a reduction in expression of *Sonic hedgehog* and *Twist* but derepresses expression of the bone morphogenetic protein-4 gene. These results are the first evidence that vertebrate NF- κ B proteins act to transmit growth factor signals between the ectoderm and the underlying mesenchyme during embryonic limb formation.

The avian limb is a classic model system that is used to study pattern formation, cell communication, proliferation, and differentiation. The transcription factor complex Rel/NF- κ B is an ideal tertiary messenger for communicating signals necessary for these functions. We have examined the expression of *c-rel* messenger RNA throughout limb bud development. This mRNA is first detected in the chick limb bud at stage 15/16 (ref. 2), before the appearance of the AER. Expression of *c-rel* remains strong within the distal compartment during limb bud outgrowth (Fig. 1a, b). As the digits form, *c-rel* mRNA levels begin to decrease within the mesenchyme, persisting only in regions adjacent to the cartilage anlage (Fig. 1c, d). By stage 34, *c-rel* is no longer detected. NF- κ B RelA is similarly expressed in mice (P.B.B. and L.D.K., unpublished observations and Supplementary Information). This expression pattern suggests a role for transcriptional regulation by Rel/NF- κ B within the developing limb.

Classical studies showed that AER ablation results in limb truncation³. The high levels of *c-rel* mRNA in the limb bud progress zone indicate that the AER may influence *c-rel* expression. Excision of the AER from a stage-20 right forelimb results in a decrease in *c-rel* expression within 4 h, with complete loss of expression by 24 h, whereas its expression is normal in the unmanipulated contralateral limb (Fig. 1e, f, h). Fibroblast growth factors (FGFs)-2, -4 and -8 are secreted by, and can functionally replace, the AER^{4–7}. To determine whether *c-rel* transcription is responsive to AER-derived FGFs, a bead loaded with recombinant FGF-4 was applied to the limb mesenchyme after AER excision. FGF-4 is capable of maintaining *c-rel* mRNA expression in an AER-deficient limb bud (Fig. 1g).

To examine the role of NF- κ B proteins in the limb bud, we employed a transdominant inhibitor of NF- κ B activity, I κ B- α ΔN (ref. 8), which lacks the first 40 amino acids containing the critical serine residues that have been implicated in the signal-induced

degradation of I κ B- α . The expressed I κ B- α ΔN protein associates with cytoplasmic Rel/NF- κ B complexes, effectively sequestering NF- κ B in the cytoplasm^{9,10}. Two strategies were used to deliver the I κ B- α ΔN *in ovo*. First, replication-competent RCAS retroviruses were used to infect chick embryos *in ovo*. Injection of RCAS- α ΔN into a stage-10 chick pre-limb field results in either a high percentage of death *in ovo* or severe reduction and truncation of the forelimb (Table 1 and Fig. 2b). We suggest that the excessive embryonic death may be due to extensive spread of virus in tissues outside the limb, as suggested by the spread of RCAS-alkaline phosphatase (Fig. 2a). A second approach was to use replication-deficient adenoviral vectors to deliver the I κ B- α ΔN (rAd- α ΔN) transgene to specific regions within the limb field. Before stage 21, Rel/NF- κ B activity is needed for proper limb morphogenesis (Table 1). We observed significant morphological defects in 70% of the embryos injected with rAd- α ΔN. These defects ranged from a

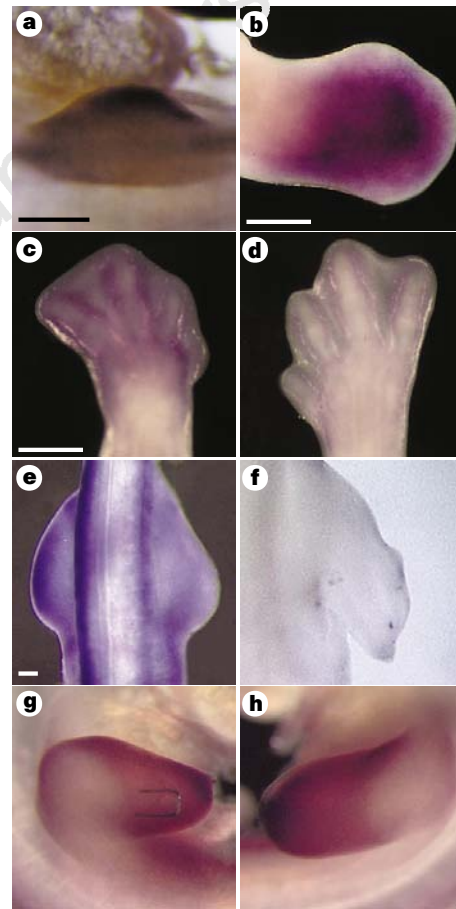


Figure 1 *c-rel* mRNA is expressed in discrete spatial and temporal patterns directed by AER-derived signals. Whole-mount *in situ* hybridization for avian *c-rel* mRNA was performed on chicks stage 20 (a), 25 (b), 30 (c) and 33 (d). a, *c-rel* mRNA is detected throughout the wing bud with highest expression in the progress zone. b, As the wing bud matures, high *c-rel* mRNA expression remains within the distal mesenchyme. No expression is seen in the AER. c, In stage 30 hindlimbs, expression of *c-rel* mRNA is limited to mesenchyme adjacent to the developing cartilage. d, Low-level expression of *c-rel* mRNA is seen around the perichondrial regions of the digits, with no detectable staining in the distal mesenchyme. e, *c-rel* mRNA levels are reduced 4 h after AER removal (right limb) as compared with the unmanipulated limb (left), and are completely lost within 24 h after surgery (f). g, *c-rel* mRNA expression is restored to an AER-deficient limb into which a bead labelled with recombinant FGF-4 has been implanted (platinum staple is seen). Partial restoration of limb outgrowth is observed. h, Normal expression of *c-rel* mRNA in the unmanipulated limb. Scale bars are (a) 500 μ m, (b) 700 μ m, (c, d) 1 mm and (e–h) 200 μ m. Darker areas indicate *c-rel* expression.

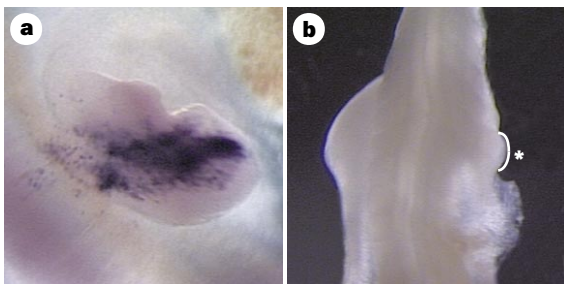


Figure 2 Sequestration of NF-κB activity leads to a reduction in limb outgrowth along both the proximo-distal and the antero-posterior axes. **a**, Control injections using RCAS-alkaline phosphatase into a stage-10 forelimb field show viral expression (dark areas) within the limb at stage 24. **b**, RCAS-αΔN produces a reduction in limb outgrowth. RCAS-αΔN was injected once into a stage-10 right-forelimb field and the embryo was then reincubated until stage 20. The infected limb is outlined in white and indicated by an asterisk. There is extreme reduction along the antero-posterior and proximo-distal axes.

reduction in size or a dorsal bend alone, to, most frequently, a combination of reduction and dorsal bend with loss of distal elements and a dysmorphic AER (Fig. 3 and Table 1). Loss of individual digits, fusion of distal cartilage elements, and distorted/reduced cartilage structures were also commonly observed phenotypes, shown in Fig. 3. To the best of our knowledge, the dorsal bend is unlike any previously described mutation and remains unexplained. These results show a temporal window (approximate, stages 13–21) in which inhibition of Rel/NF-κB activity grossly alters limb outgrowth, rotation, and digit formation, suggesting a requirement for NF-κB in limb morphogenesis.

To determine whether NF-κB activity is necessary within a defined region in the limb bud, specific sites within stage 17–21 limb buds were infected with rAd-αΔN. The zone of polarizing activity (ZPA), a region of posterior mesenchyme necessary for antero-posterior patterning, the anterior side of the limb bud, and the progress zone were infected individually. Although the percentage of embryos exhibiting a severe phenotype was slightly lower than when the entire limb field was injected, the same range in phenotypes was observed whether infection was directed to the posterior, anterior, or distal mesenchyme (Table 1). This suggests that rAd-αΔN interrupts mesenchymal cell function.

Injections with adenoviral dilution buffer, empty recombinant adenovirus virions, or recombinant adenovirus containing wild-type IκB-α does not produce any alteration in the injected limb (data not shown). The wild-type pattern of *c-rel* mRNA expression is observed in the contralateral uninjected limb bud, whereas a reproducible decrease in *c-rel* mRNA expression is observed in rAd-αΔN-infected limbs. This reduction in *c-rel* mRNA levels is consistent with the observation that the *c-rel* promoter is autoregulated

Table 1 RCAS-αΔN and rAd-αΔN-infected limbs exhibit a range of phenotypic alterations depending on the developmental stage and position of infection

RCAS-ΔN					
Stage	Total infected	Dead	Limb reduced	Embryos deformed	
10	<i>n</i> = 45	25	10	10	
rAd-ΔN					
Stage	Total infected	Twisted	Reduced	Twisted and reduced	Percentage with phenotype
13–16	<i>n</i> = 29	3	6	12	72
17–21	<i>n</i> = 258	43	32	121	76
ZPA	<i>n</i> = 30	4	4	11	63
Ant.	<i>n</i> = 15	1	2	6	60
PZ	<i>n</i> = 23	5	3	8	70
Entire	<i>n</i> = 190	33	23	96	80
22–25	<i>n</i> = 25	0	0	0	0

Ant., anterior; PZ, progress zone.

by Rel/NF-κB¹¹. Limbs showing a more severe phenotype have a greater reduction in *c-rel* mRNA levels when compared with moderately twisted limb buds (P.B.B. and L.D.K., unpublished observations). This suggests that the range in phenotype and *c-rel* RNA expression is due, in part, to the extent of adenoviral infection.

Our results also indicate a link between *c-rel* expression and the AER. In current models of limb development, a positive feedback loop involving AER-derived signals and their signalling counterparts from the mesenchyme is necessary for the proliferation and differentiation of the mesenchyme and the maintenance of the AER, respectively^{12,13}. The AER is defined first histologically, as a ridge of pseudostratified columnar epithelium traversing the distal antero-posterior border of the developing limb bud, and second, it is defined molecularly as the source for FGF-2, FGF-4 and FGF-8. We used a probe for FGF-8 mRNA as well as scanning electron microscopy to visualize the AERs of infected limbs (Fig. 4). The aberrant skewing of the infected limb's AER contrasts clearly with the symmetrical line of cells seen in the uninfected contralateral limbs. Infection with rAd-αΔN results in a general loss of normal AER structure, most vividly indicated by the dorsal skewing, foreshortening and clumping of the 'AER'. Thus, we propose that sequestration of Rel/NF-κB results in an interruption of interactions between mesenchymal and epithelial cells and may alter epithelial cell differentiation and function.

To determine the mechanism by which NF-κB affects limb development, we sought downstream targets of NF-κB activity. Within RCAS-αΔN-infected limbs, expression of *Sonic hedgehog* (*Shh*) mRNA is markedly reduced (Fig. 5b). A similar reduced pattern of *Shh* mRNA expression is observed in AER-deficient limbs^{11,12}. This indicates that NF-κB may transduce the FGF-

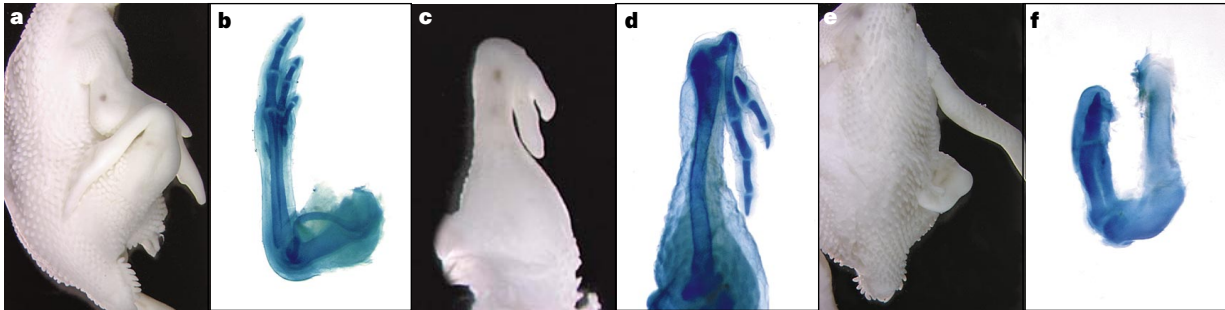


Figure 3 Sequestration of NF-κB leads to a dysmorphic limb. Three stage-35 embryos exhibiting the result of rAd-αΔN infection are shown. **a**, **c**, **e**, Demonstrate the dorsal bend and overall reduction in size of the infected right hindlimb. Subsequent cartilage staining with Alcian Blue (**b**, **d**, **f**) reveals the bent

cartilage structures and the cartilage elements. The bent phenotype is found in either the femur (**f**), the metatarsals (**d**), or the tibia (**b**). **a**, **b**, A single digit and metatarsal are absent. **c**, **d**, Two digits, two metatarsals and the tibia are absent. **e**, **f**, All digits are missing and the tibia and fibula are thickened and foreshortened.

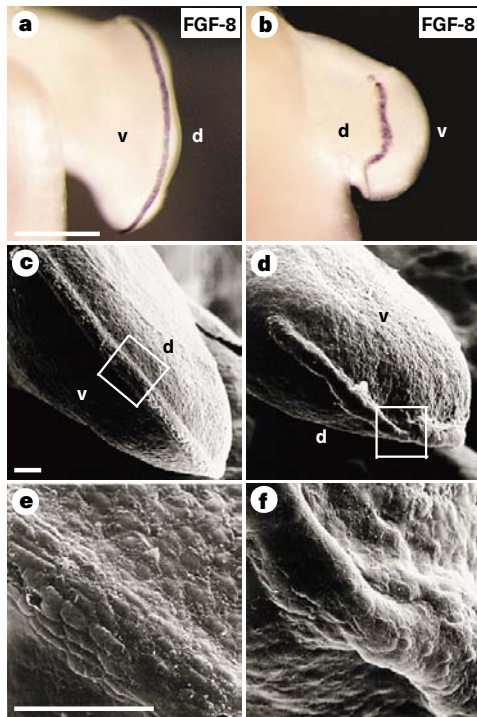
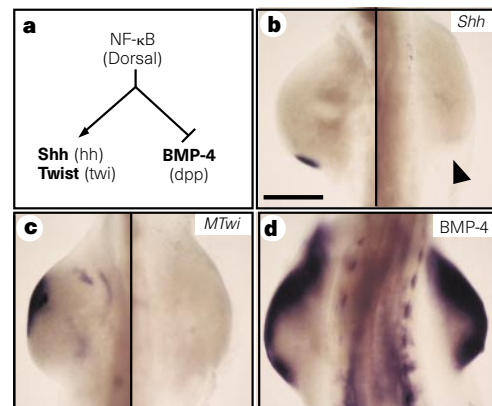


Figure 4 Inhibition of NF- κ B activity results in a dysmorphic AER. Whole-mount *in situ* hybridization was used to detect *FGF-8* mRNA expression in control and rAd- α Δ N-infected limbs. This shows structural alterations of the AER. Dorsal (d) and ventral (v) surfaces are indicated to correct for the 180° twist of the infected limbs. **a**, *FGF-8* is expressed throughout the AER in an uninterrupted, smooth, dark arc. **b**, In infected limbs, *FGF-8* expression is foreshortened along the antero-posterior aspect and the AER is highly convoluted, skewed towards the dorsal surface, and thickened in regions. **c**, Scanning electron micrograph of an uninfected left limb of a stage-28 embryo at approximately $\times 115$ magnification emphasizes the smooth symmetry of a wild-type AER. The anterior AER border extends out of the micrograph. **d**, In contrast, the AER of the infected limb of the same embryo is irregular and convoluted with areas of thickened cell masses and other regions almost lacking recognizable AER. The AER is skewed dorsally and abruptly halts at the anterior border. **e**, Magnification ($\times 900$) of the boxed region in **c**. The highly articulated columnar AER and its smooth fusion into the keratinized squamous epithelial cells of the dorsal surface ectoderm is seen. **f**, Magnification ($\times 900$) of the infected limb from the boxed region in **d** shows the loss of normal AER cellular architecture. Scale bars are (**a**, **b**) 700 μ m and (**c**–**e**) 50 μ m.

Figure 5 Inhibition of NF- κ B activity leads to a reduction of *Shh* and *Twist* mRNA expression while derepressing *BMP-4* gene expression. **a**, The *Drosophila* homologue of Rel/NF- κ B, Dorsal, transcriptionally activates the expression of *twist* (*twi*) and *hedgehog* (*hh*) while repressing *dpp* transcription. The *Drosophila* genes/proteins are shown in parentheses; the chick proteins are not. **b**–**d**, Whole-mount *in situ* hybridization of stage-20 embryos that have been infected with RCAS- α Δ N at stage 10 into the right forelimb field. The infected limbs are shown in the right panel and the contralateral limb in the left panel. **b**, The infected limb exhibits a significant reduction in *Shh* mRNA levels. **c**, *Twist* mRNA (*MTwi*) is not detectable in the infected limb. **d**, *BMP-4* expression is upregulated throughout the infected mesenchyme. Embryos are representatives of 3–4 independent infections. Scale bar represents 500 μ m. Black arrowhead indicates absence of *Shh* expression.



mediated signals that regulate *Shh* expression and may participate in the formation of the antero-posterior organizing centre. In *Drosophila*, the morphogen Dorsal directly activates and represses the transcription of *twist* and *decapentaplegic* (*dpp*), respectively (Fig. 5a)^{14–17}. A vertebrate *twist* homologue is expressed in the nuclei of mesenchymal limb cells¹⁸, and is strongly expressed along the distal margin of a stage-20 chick limb (Fig. 5c). *Twist* mRNA is absent in RCAS- α Δ N-infected limbs (Fig. 5c). This is interesting in light of the human mutation in *Twist* in Saethre-Chotzen syndrome, in which craniofacial and limb anomalies are seen. Although Rel/NF- κ B factors are expressed in discrete temporal and spatial patterns in both the limb and the pharyngeal arches, we speculate that a function of the κ B factors is to modulate *Twist* gene expression during development. Other genes implicated in limb patterning (for example, cHoxA10, cHoxA11, cHoxD9, cHoxD10, and cHoxD13) are not affected by NF- κ B sequestration (P.B.B. and L.D.K., unpublished observations). In contrast, the expression of the vertebrate homologue of *dpp*, bone morphogenetic protein-4 (BMP-4), is upregulated in RCAS- α Δ N-infected limbs (Fig. 5d). Because BMP-4 is required for mesoderm differentiation¹⁹, regulation of mesenchymal programmed cell death²⁰, and the development of cartilage²¹, the data that IkB- α Δ N-infected/BMP-4-overexpressing limbs are truncated and exhibit dysmorphic skeletal elements support previous observations.

Our results indicate that Rel/NF- κ B transcriptionally regulates the expression of factors that are necessary for the determination of positional patterning, cellular identity and survival. Just as the AER is required to maintain the proliferative, undifferentiated state of the progress zone, so the mesenchyme is necessary for maintenance of the structure and function of the AER²². One example of mesenchymal-epithelial interactions details the feedback loop between *Shh* and FGF-4. FGFs induce the expression of *Shh* mRNA within the ZPA, and *Shh* then signals for the continued expression of FGF-4 within the AER^{12,13}. The introduction of IkB- α Δ N markedly affects both AER morphology and *Shh* expression. We propose that NF- κ B activity is critical for mesenchymal maintenance of the AER and that the phenotypes observed upon sequestration of Rel/NF- κ B by a transdominant inhibitor are a consequence of a failure in mesenchymal-epithelial communication. □

Methods

Whole-mount RNA *in situ* hybridization and RNA probes. Whole-mount *in situ* hybridization was performed as described²³. Digoxigenin-labelled antisense RNA probes for avian *FGF-8*, *Shh*, *MTwi* and *BMP-4* were prepared as described^{7,24–26}. To produce a *c-rel* antisense RNA probe, 1.8 kb chick *c-rel* cDNA were subcloned into pBlueScript II (SK-), linearized using *Sma*I, transcribed with T3 RNA polymerase, and digoxigenin-labelled according to manufacturer's specifications (Boehringer).

AER excision and bead implantation. Fertilized chicken eggs (SPAFAS) were incubated at 39 °C for ~72–80 h. After staining with a dilute Nile Blue solution, the AER from the right forelimb was surgically excised and the embryo was incubated for the time specified and was then fixed in 4% paraformaldehyde. Heparin acrylic beads, of ~200 µm in diameter, were soaked in 1 mg ml⁻¹ FGF-4 (R&D Systems) for 1 h at ambient temperature. One bead was stapled onto the posterior mesenchyme of a stage-20 forelimb from which the AER was excised.

Virus preparation and infections. An *EcoRI* fragment of BS(Sk)-IκB-αΔN of 0.9 kb was subcloned into a Cla12-Nco adaptor plasmid and then into RCAS-BP(A)²⁷. RCAS viral stocks were produced as described²⁸. The vector rAd-αΔN was constructed by ligating the 0.9-kb *Acc651* fragment of pBS(SK)-IκB-αΔN into the *Acc651* site of vector pAC. Identical construct formation was used for wild-type IκB-α, pAC-IκBα. We used a modified procedure from ref. 29 to prepare adenoviruses; additional details will be supplied upon request. Fertilized chicken eggs were incubated at 39 °C for 36–96 h. The eggs were windowed and the region to be infected stained with Nile Blue. Virus was loaded into a capillary pipet, attached to a picospritzer II and delivered at 10 p.s.i. in three pulses of 30 ms. For injections within the entire limb, virus was injected at 12–16 locations, depending on the limb size. For infection of a specific region, for example, the ZPA, eight injections were performed within that region. The egg was reincubated for 36 h or 7 days before being fixed in 4% paraformaldehyde for *in situ* hybridization or in 5% trichloroacetic acid (TCA) for cartilage staining, respectively.

Cartilage staining. TCA-fixed embryos were stained overnight in 0.075% Alcian Blue in acid alcohol, partially destained in 2% aqueous KOH, dehydrated in 100% ethanol and cleared in methyl salicylate.

Scanning electron microscopy. Samples were fixed in 3% glutaraldehyde buffered to pH 7.3 with sodium cacodylate. Following post-fixation in OsO₄, they were dehydrated, critical-point dried, and sputter-coated with gold before viewing in a Hitachi S-500 scanning electron microscope.

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- Nüsslein-Volhard, C., Lohs-Schardin, M., Sander, K. & Cremer, C. A dorsal-ventral shift of embryonic primordia in a new maternal-effect mutant of *Drosophila*. *Nature* **283**, 474–476 (1980).
- Hammer, V. & Hamilton, H. L. A series of normal stages in the development of the chick embryo. *J. Exp. Morphol.* **88**, 49–92 (1951).
- Summerbell, D. A quantitative analysis of the effect of the excision of the AER from the chick limb bud. *J. Embryol. Exp. Morphol.* **32**, 651–660 (1974).
- Fallon, J. F. et al. FGF-2: apical ectodermal ridge growth signal for chick limb bud development. *Science* **264**, 104–107 (1994).
- Niswander, L., Tickle, C., Vogel, A., Booth, I. & Martin, G. R. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579–587 (1993).
- Vogel, A., Rodriguez, C. & Izpisua-Belmonte, J. C. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737–1750 (1996).
- Crossley, P. H., Minowada, G., MacArthur, C. A. & Martin, G. R. Roles for FGF8 in the induction, initiation and maintenance of chick limb development. *Cell* **84**, 127–136 (1996).
- Inoue, J.-i. et al. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. *Proc. Natl Acad. Sci. USA* **89**, 4333–4337 (1992).
- Brockman, J. A. et al. Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. *Mol. Cell. Biol.* **15**, 2809–2818 (1995).
- Treancker, E. B.-M. et al. Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J.* **14**, 2876–2883 (1995).
- Grumot, R. J., Richardson, I. B., Gaff, C. & Beronakis, S. Rel/NF-κB nuclear complexes that bind κB sites in the murine c-rel promoter are required for constitutive c-rel transcription in B cells. *Cell Growth Differ.* **4**, 731–743 (1993).
- Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. & Tabin, C. Sonic hedgehog and FGF-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993–1003 (1994).
- Niswander, L., Jeffrey, S., Martin, G. R. & Tickle, C. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609–612 (1994).
- Jiang, J., Kosman, D., Ip, Y. T. & Levine, M. The dorsal morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Dev.* **5**, 1881–1891 (1991).
- Pan, D., Huang, J.-D. & Courey, A. J. Functional analysis of the *Drosophila* *twist* promoter reveals a dorsal-binding ventral activator region. *Genes Dev.* **5**, 1892–1901 (1991).
- Huang, J. D., Schwytter, D. H., Shirokawa, J. M. & Courey, A. J. The interplay between multiple enhancer and silencer elements defines the pattern of decapentaplegic expression. *Genes Dev.* **7**, 694–704 (1993).
- Schwytter, D. H., Huang, J. D., Dubnicoff, T. & Courey, A. J. The decapentaplegic promoter region plays an integral role in the spatial control of transcription. *Mol. Cell. Biol.* **15**, 3960–3968 (1995).
- Gitelman, I. Twist protein in mouse embryogenesis. *Dev. Biol.* **189**, 205–214 (1997).
- Winnier, G., Blessing, M., Labosky, P. A. & Hogan, B. L. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9**, 2105–2116 (1995).
- Yokouchi, Y. et al. BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development* **122**, 3725–3734 (1996).
- Duprez, D. et al. Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57**, 145–157 (1996).
- Saunders, J. W., Jr & Gasseling, M. T. In *Epithelial-Mesenchymal Interaction* (eds Fleischmayer, R. & Billingham, R. E.) 78–97 (Williams and Wilkins, Boston, 1968).
- Ros, M., Lyons, G. & Fallon, J. Spatial and temporal analysis of homeobox genes expressed in chick limb buds by whole mount *in situ* hybridization. *Prog. Clin. Biol. Res.* **383A**, 79–87 (1993).

- Echelard, Y. et al. *Sonic hedgehog*, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417–1430 (1993).
- Winnier, G. E., Hargett, L. & Hogan, B. L. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. *Genes Dev.* **11**, 926–940 (1997).
- Wall, N. A. & Hogan, B. L. Expression of bone morphogenetic protein-4 (BMP-4), bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-8 (FGF-8) and sonic hedgehog (SHH) during branchial arch development in the chick. *Mech. Dev.* **53**, 383–392 (1995).
- Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. & Sutcliffe, P. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004–3012 (1987).
- Morgan, B. A. & Fekete, D. M. Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol.* **51**, 185–218 (1996).
- Becker, T. C. et al. Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* **43**, 161–176 (1994).

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A proteolytic system that compensates for loss of proteasome function

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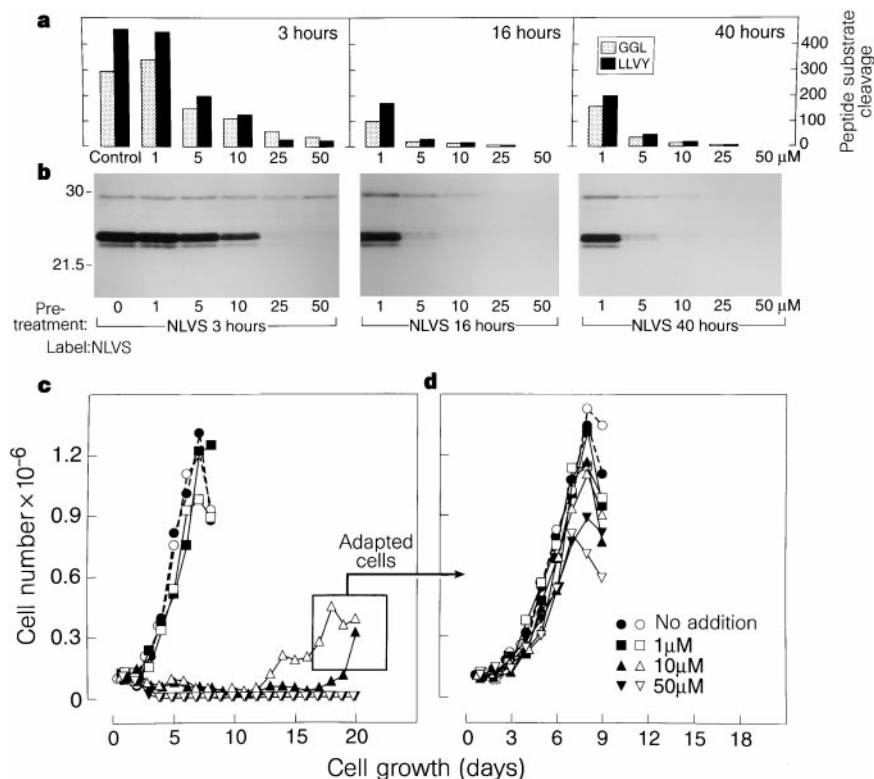
Proteolysis is essential for the execution of many cellular functions. These include removal of incorrectly folded or damaged proteins¹, the activation of transcription factors², the ordered degradation of proteins involved in cell cycle control³, and the generation of peptides destined for presentation by class I molecules of the major histocompatibility complex⁴. A multisubunit protease complex, the proteasome⁵, accomplishes these tasks. Here we show that in mammalian cells inactivation of the proteasome by covalent inhibitors allows the outgrowth of inhibitor-resistant cells. The growth of such adapted cells is apparently maintained by the induction of other proteolytic systems that compensate for the loss of proteasomal activity.

Proteasomal function can be inhibited pharmacologically by covalent or non-covalent modification of proteasomal β-subunits. Peptide aldehydes⁶ act by forming a reversible hemiacetal adduct with Thr1 on catalytically active β-subunits^{6,7}, whereas the natural product lactacystin irreversibly alkylates Thr1^{8–11}. An amino-terminally modified tri-leucine vinyl sulphone, NIPL₃-VS (NLVS), is a selective, covalent inhibitor that penetrates cell membranes and can be used to inhibit proteasomes in living cells¹². NLVS and related peptide vinyl sulphones selectively and covalently modify all three catalytically active (X, Y and Z) β-subunits, as well as the γ-interferon-inducible β-subunits (LMP2, LMP7 and MECL-1), with little or no crossreaction with non-proteasomal proteases¹².

EL-4 lymphoma cells, maintained in the presence of 10 µM NLVS, have drastically reduced proteasomal activity and die after 24–48 hours (Fig. 1). Homogenates from such EL-4 cells show

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Figure 1 Covalent modification of proteasomes in EL-4 cells cultured in the presence of NLVS blocks enzymatic activity. The block in proteasomal activity inhibits cellular proliferation, but a minor population of EL-4 cells adapts to lethal concentrations of NLVS after prolonged culture. **a**, EL-4 cells were incubated in the presence of unlabelled NLVS for the indicated times and cellular homogenates of these cells were tested against the fluorogenic peptide substrates suc-LLVY-MCA (grey bars) and Z-GGL-MCA (black bars), showing progressive loss of enzymatic activity in a concentration- and time-dependent manner. **b**, Cellular homogenates in **a** were labelled with [125 I]NLVS. **c**, EL-4 cells (100,000 cells per well) were cultured with the indicated concentrations of NLVS and counted daily. **d**, Cells recovered from 10 μ M NLVS were adapted to grow in the presence of 50 μ M NLVS by gradually increasing the concentration of NLVS during culture.



reduced hydrolysis of proteasome-preferred fluorogenic peptide substrates (Fig. 1a). Moreover, their proteasomal β -subunits are refractory to modification with [125 I]NLVS (Fig. 1b). The lowest concentration of NLVS required to block proteasomal activity (5–10 μ M) also inhibits the growth of EL-4 cells (Fig. 1a–c); the toxic and inhibitory effects of NLVS depend on its tri-leucine sequence, indicating that the toxicity is due to inhibition of the proteasome (data not shown). In contrast, prolonged exposure of EL-4 cells to the corresponding tri-leucine peptide aldehyde inhibitor (Z-L₃-H) is toxic at 0.5–1.0 μ M, a concentration at which there is only minimal inhibition of proteasomal proteolysis^{4,12}.

Although most EL-4 cells cultured in the presence of 10 μ M NLVS die (Fig. 1), after 2–3 weeks a minor population of EL-4 cells recovers and grows (termed adapted cells; Fig. 1c, d). Limiting dilution indicated a frequency of growth of ~1 in 300 cells in the presence of 10 μ M NLVS, whereas 50 μ M NLVS did not allow any recovery of cell growth (data not shown; Fig. 1c). This frequency of adaptation rules out mutation as the cause of resistance to NLVS, as does the observation that removal of NLVS allows recovery of proteasomal activity after 24 h of culture (data not shown). EL-4 cells, adapted to grow in up to 50 μ M NLVS by gradually increasing the concentration of the inhibitor, had a growth rate similar to normal EL-4 cells. In overgrown cultures, adapted EL-4 cells died more rapidly than control cells, which could be due to effects on cellular metabolism by high concentrations of NLVS when nutrients are limiting (Fig. 1d).

Exposure of intact cells to [125 I]NLVS showed labelling of control but not of adapted EL-4 cells, indicating prior modification of proteasomal β -subunits. To rule out the possibility that a change in membrane permeability of adapted cells prevents access of NLVS to the proteasome, proteolytic activity was monitored in cellular homogenates. Hydrolysis of proteasome substrates Z-GGL-MCA, Suc-LLVY-MCA (both substrates for the chymotrypsin-like activity) and Z-LLE- β NA (substrate for the PGPH (post glutamyl peptide hydrolysing activity) was reduced in adapted EL-4 cells to about 10–25% of control values, and labelling of proteasomal β -subunits by [125 I]NLVS was largely absent (data not shown). Gel

filtration and the assay of individual fractions for hydrolysis of the Suc-LLVY-MCA substrate showed a characteristic pattern of 20S proteasome (Fig. 2a: main peak fractions, 20–25) and 26S proteasome (Fig. 2a: shoulder fractions 16–19) activities in EL-4 cells, whereas very little activity (<10%) remained in extracts prepared from adapted EL-4 cells (Fig. 2a). Upon addition of [125 I]NLVS, we observed labelling of proteasomal β -subunits in fractions from control cells, corresponding to the peak of Suc-LLVY-MCA hydrolytic activity, but no labelling was detectable in fractions from adapted cells (Fig. 2b). These combined results indicate that the reduced proteasomal activity in adapted cells is attributable to covalent modification of β -subunits by NLVS.

We immunoprecipitated proteasomes from [35 S]methionine-

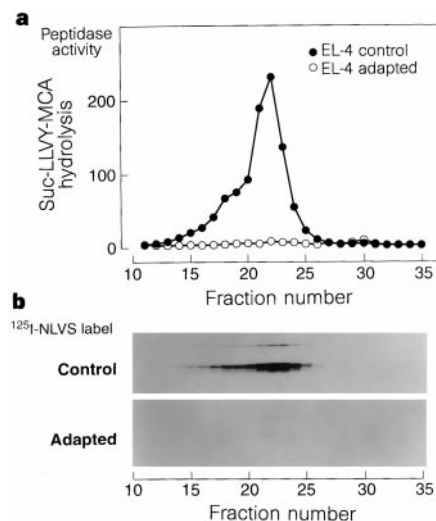


Figure 2 Peptidase activity from EL-4-adapted and control fractions resolved by gel filtration (see Methods). Cytosolic fractions were centrifugated at high speed and the pellets were resuspended and fractionated on a Superose-6 column. Each fraction was tested for peptidase activity (**a**) and labelled with [125 I]NLVS (**b**).

labelled control and adapted EL-4 cells with an antiserum against the proteasomal C9 α -subunit. Analysis by two-dimensional SDS-PAGE showed the characteristic pattern of α - and β -subunits¹³. The relative intensities of the spots corresponding to β -subunits varied slightly, but the labelling pattern was comparable for EL-4 and adapted EL-4 cells (Fig. 3a, b). The most obvious change was a comigration of LMP7 and LMP8 in proteasomes from adapted cells due to a shift in mobility of NLVS-modified LMP7 (Fig. 3b; indicated as LMP7*). This was confirmed *in vitro* by showing that NLVS-treatment of isolated proteasomal subunits from EL-4 cells also resulted in comigration of NLVS-modified LMP7 with LMP8 (Fig. 3c, d).

Proteasomal proteolysis has been implicated in the generation of major histocompatibility complex (MHC) class I bound peptides^{4,14–17}. Exposure of cells to either peptide aldehydes or lactacystin causes a temporary deficiency in the supply of peptides for MHC class I molecules^{4,16}, leading to a reduction in the amount of assembled MHC class I products. In EL-4 cells, newly synthesized class I heavy chains rapidly form stable complexes with β_2 -microglobulin (β_2 m) and acquire complex-type glycans (Fig. 4a, d). In EL-4 cells treated with NLVS, there is a substantial reduction in the formation of assembled MHC class I complexes. As reported for L929 cells¹⁸, we do not observe complete inhibition of MHC class I assembly and transport, suggesting that non-proteasomal proteases may contribute a fraction of MHC class I-presented peptides in these cells (Fig. 4). Addition of MHC-binding peptide to cell lysates restored the recovery of assembled MHC class I molecules that lack terminal glycan modifications, which indicates an arrest of intracellular transport caused by a shortage of MHC ligands (Fig. 4b, e). However, NLVS-adapted EL-4 cells displayed almost normal levels of stable and therefore peptide-loaded MHC class I molecules which indicates that there is a non-proteasomal source of MHC ligands (Fig. 4).

In non-adapted EL-4 cells treated with the proteasome inhibitor NLVS, degradation of ubiquitinated proteins is inhibited, as shown by an increase in ubiquitin-conjugated material (relative molecular masses of 50K–200K) revealed by immunoblotting (data not shown). Staining the DNA of these cells with propidium iodide showed an increased fraction of cells in the G2/M (41% in G2) phase of the cell cycle in comparison with control cells (23% in G2),

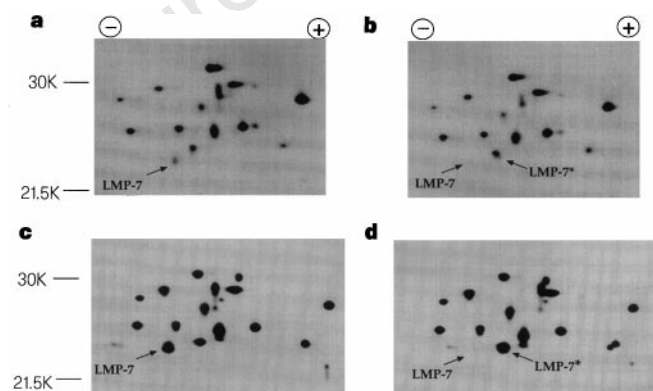


Figure 3 Synthesis and assembly of proteasome subunits is normal in adapted cells. **a–d**, Adapted and control EL-4 cells were pulse-labelled for 1 h and chased for 24 h. Proteasomes were immunoprecipitated from either normal EL-4 cells (**a**) or adapted EL-4 cells (**b**) by using an anti-C9 (α -subunit) polyclonal antiserum. **c, d**, EL-4 proteasomes were precipitated using a rabbit polyclonal antiserum raised against purified mouse proteasomes. These proteasomes were either not treated (**c**) or treated *in vitro* with 10 μ M unlabelled NLVS for 2 h (**d**). Samples were separated by two-dimensional non-equilibrium pH gradient electrophoresis and subunits visualized by fluorography. Note that the relative intensities of spots are the same for control and adapted cells, except for LMP-7 (NLVS-modified LMP7 comigrating with LMP8 is indicated as LMP7*). The spot corresponding to LMP7 was identified by its relative molecular mass and isoelectric point¹³.

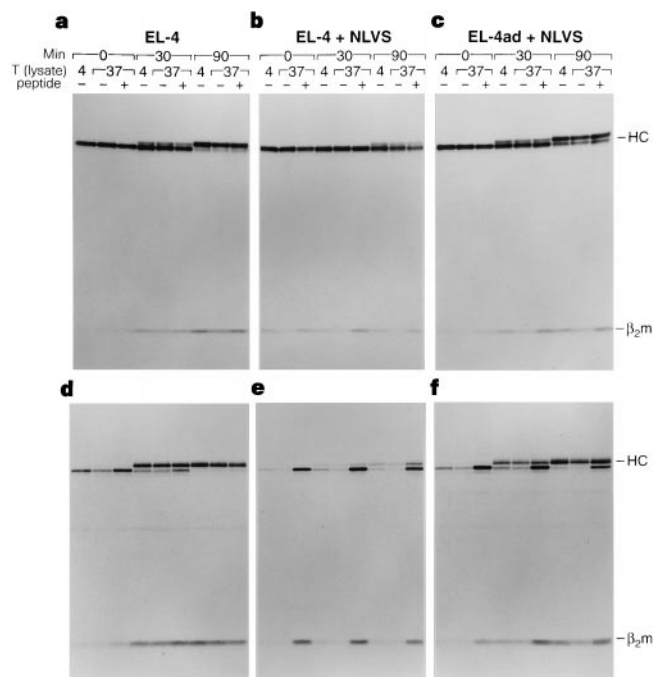


Figure 4 Adapted EL-4 cells generate ligands for stable assembly and intracellular transport of MHC class I molecules. Cells were pulsed with [³⁵S] methionine for 10 min and chased for the indicated times. The cells used were EL-4 (**a, d**), NLVS-treated EL-4 (**b, e**) and NLVS-adapted EL-4 (**c, f**). To assess total levels of class I heavy chain, regardless of peptide occupancy or state of assembly, we used an antiserum (anti-p8) against the cytoplasmic tail of H-2K^b (**a–c**). To detect properly conformed H-2K^b molecules, we used the monoclonal antibody Y3 (**d–f**). Lysates were exposed to 37°C for 20 min in the presence or absence of 10 μ M of an H-2K^b-binding peptide (SIINFEKL), or were kept at 4°C. Only peptide-stabilized class I molecules maintain their conformation (Y3-reactive) at 37°C. Bands corresponding to the heavy chain (HC) and β_2 m are indicated.

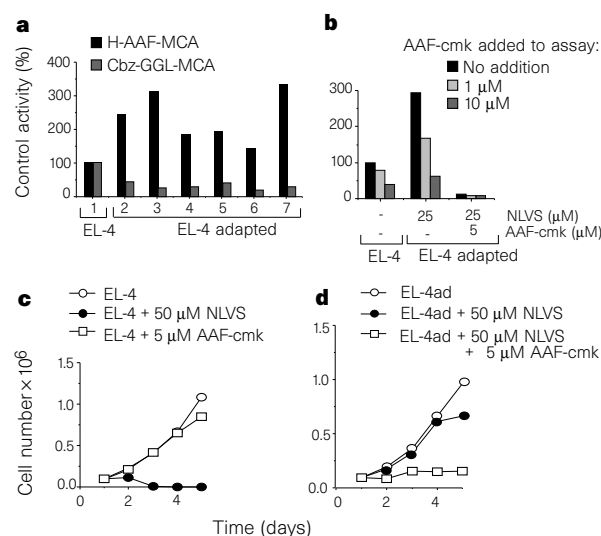


Figure 5 AAF-MCA hydrolysis is increased in adapted cells. **a**, Cytosol prepared from independent cultures (bars 2–7) of adapted EL-4 cells shows increased hydrolysis of AAF-MCA peptide substrate and decreased hydrolysis of Cbz-GGL-MCA (proteasome substrate) compared with EL-4 (bar 1). **b**, The cytosolic activity that cleaves AAF-MCA is blocked by AAF-CMK both *in vivo* and *in vitro*. **c, d**, Inhibition of AAF cleavage is growth-inhibitory for adapted EL-4 cells (**d**) but not for control EL-4 cells (**c**).

consistent with proteasomal proteolysis being required at the G2/M–G1 transition^{3,19–21}. In adapted EL-4 cells growing in the presence of NLVS, no cells accumulated in the G2/M phase (25% in G2), and the levels of ubiquitinated proteins were comparable to those in untreated EL-4 cells (data not shown). Thus, in adapted EL-4 cells, cell-cycle progression (Fig. 1d) and degradation of ubiquitinated proteins continue in the absence of normal proteasomal function.

The occurrence of a large ($M_r > 1,000K$) protease complex in *Thermoplasma*, termed tricorn protease²², inspired us to examine the possibility of similar large complexes in adapted cells that could functionally replace proteasomal activity. Tricorn protease is resistant to proteasomal inhibitors and, characteristically, hydrolysis of the AAF-MCA substrate requires a free amino terminus. We indeed observed a remarkable increase in AAF-hydrolysing activity in adapted compared with normal EL-4 cells (Fig. 5a).

Although the active site of the tricorn protease has not yet been identified, it may contain a serine nucleophile²². As chloromethylketones are potent irreversible inhibitors of serine proteases²³, we synthesized AAF-chloromethylketone (AAF-CMK) and found that it blocked AAF-hydrolysing activity in a dose-dependent manner, achieving >80% inhibition *in vitro* at 10 μM AAF-CMK (Fig. 5b) without inhibiting proteasomal activity. If the AAF-MCA hydrolysing activity in adapted cells can compensate for loss of the proteasome, then inhibiting this activity should prevent growth of adapted EL-4 cells. There was no effect of 5 μM AAF-CMK on the growth of normal EL-4 cells, whereas proliferation of adapted EL-4 cells was inhibited (Fig. 5c, d). The AAF-MCA cleaving activity must therefore be part of a protease or protease complex that contributes to or is required for survival of adapted cells.

To distinguish the induced protease activity, from the proteasome, we isolated material by centrifugation of cytosol for 5 hours at 100,000g, fractionated this by gel filtration, and tested each fraction for its capacity to hydrolyse AAF-MCA (Fig. 6; solid lines). The peak of AAF-MCA hydrolysing activity resolved from the void volume and eluted before the 20S and 26S proteasomes (Fig. 6). The AAF-hydrolytic activity is thus associated with a complex larger than the proteasome. This activity cannot be inhibited by NLVS, whereas addition of 10 μM AAF-CMK blocks it completely (data not shown, and Fig. 5). Analysis by SDS–PAGE and silver staining showed that in control cells there was a cluster of proteasomal β -subunit-sized polypeptides of 20K–30K in fractions that cleave the proteasome substrate LLVY-MCA, and in adapted cells there was a complex set of polypeptides in the fractions that cleave AAF-MCA.

Proteasomal proteolysis can usually not be compromised without inhibiting proliferation^{5,24} (Fig. 1). We have described the outgrowth of cells that, upon prolonged exposure to the proteasome inhibitor NLVS, appear to be resistant to the inclusion of otherwise toxic concentrations of this inhibitor. In these adapted cells there is

an increase in enzymatic activity capable of hydrolysing AAF-MCA which appears to be essential for the cells to grow when proteasomal activity is blocked. This activity is separable from both 26S and 20S proteasomes in specificity, size and inhibition profile. We propose that a minority of cells express auxiliary proteases. In the absence of proteasomal inhibition, these cells have no obvious growth advantage and are not represented in appreciable numbers. In the continuous presence of NLVS, however, the rare cells that express more AAF-hydrolysing activity are spared and recover to expand after two weeks of culture, relying at least partly on AAF-hydrolysing activity to do so.

We conclude that proteasomes can be replaced functionally by other protease activities, which raises questions about the fate of ubiquitin-conjugated proteins in adapted cells, on the mechanisms used by adapted cells to regulate their cell cycle, and on the possible involvement of non-proteasomal cytosolic proteases in MHC class I-restricted antigen presentation. Destruction of cyclins and cyclin-dependent-kinase inhibitors has almost invariably been attributed to the ubiquitin–proteasomal system^{3,25–27}. The detailed molecular characterization of the activities that allow adapted cells to survive will be a challenge. □

Methods

Cells and cell culture. The mouse lymphoma cell line EL-4 was maintained in Dulbecco's modified Eagle's or RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. Adapted EL-4 cells were maintained in the presence of 50 μM 5-iodo-4-hydroxy-nitrophenyl acetyl-leucyl-leucyl-leucine vinyl sulphone¹².

Inhibitors. [¹²⁵I]NIP-L₃-VS and NIP-L₃-VS (NLVS) were synthesized as described¹². All inhibitors were added to lysates and to cells by dilution of a dimethylsulphoxide (DMSO) stock solution such that the final concentration of DMSO was 1%.

Antibodies. Anti-C9 is a monoclonal antibody against a proteasomal α -subunit in assembled proteasomes; rabbit anti-mouse proteasome is an antiserum directed against assembled mouse proteasomes (gift from J. Monaco)¹³; Y3 (ref. 28) is a monoclonal antibody that recognizes the $\alpha 1/\alpha 2$ domain of properly folded mouse class I heavy chains; anti-P8 is a rabbit antiserum specific for exon 8 in the cytoplasmic tail of H-2K^b mouse MHC class I heavy chains; and anti-ubiquitin is a polyclonal antiserum reactive against ubiquitin and ubiquitin conjugates (gift from A. L. Haas).

Growth curves. Cells (100,000 in 1 ml medium) were grown in the presence of inhibitor or DMSO (1%) in 24-well plates. Cells were resuspended daily, 20- μl aliquots were removed and mixed with trypan blue dye (0.1%). Cells that excluded the dye were considered viable and were counted.

Labelling of proteasomes with [¹²⁵I]NIP-L₃-VS. Cells (1.5×10^6) or lysates (50 μg unless otherwise indicated) were labelled with [¹²⁵I]NLVS, diluted to a final concentration of 1.8×10^4 Bq ml⁻¹ in tissue culture medium (cells) or buffer I (lysates; 50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl₂, 2 mM ATP). Samples were incubated at 37 °C for 2 h then 1 $\times$ (cells) or 4 $\times$ (lysates) SDS-sample buffer was added. Proteins were separated by SDS–PAGE.

Preparation of lysates from control EL-4, NLVS-treated or adapted cells. Cells were washed in cold PBS, then in buffer I, and pelleted. Glass beads (<106 microns, acid-washed; Sigma) equivalent to the volume of the cellular pellet were added, followed by the same volume of homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM MgCl₂, 2 mM ATP, 250 mM sucrose). Cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1,000g for 5 min, followed by 10,000g for 20 min. Protein concentration was determined using the BCA protocol (Pierce Chemical).

Fluorogenic peptide substrate assay. Peptidase activity was measured using the following fluorescent labelled peptide substrates: Suc-LLVY-MCA, Z-LLG-MCA and Suc-AAF-MCA for analysis of chymotrypsin-like activity of the proteasome; Boc-LRR-MCA for analysis of the trypsin-like activity of the proteasome; Z-LLE- β NA for analysis of the PGPH activity of the proteasome; and AAF-MCA for analysis of non-proteasomal proteolysis. Partially purified proteasomes from 5-h pellets (5 μg total protein), or total lysates (10 μg) were diluted to 100 μl in only buffer I or 50 mM Tris, pH 7.4. Inhibitors and substrates (100 μM) were added to samples as DMSO stocks. Samples were

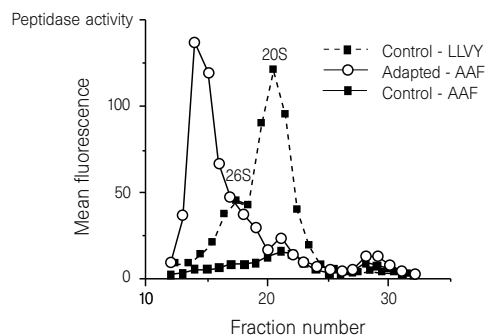


Figure 6 AAF-MCA hydrolysing activity elutes before the proteasome, indicating that it has a higher M_r . Protein fractions from differential centrifugation of cytosols (see Methods) were fractionated on a Superose-6 gel filtration column as for Fig. 2. Fractions were assayed for hydrolysis of the non-proteasomal substrate AAF-MCA (solid lines) or Suc-LLVY-MCA (dashed line, indicating the elution of proteasomal activity).

incubated at 37 °C for 45 min and the reaction was quenched with 1 ml 1% SDS. Fluorescence was measured using a Hitachi F4500 spectrofluorimeter.

Class I assembly. To monitor class I assembly, a pulse-chase experiment was done using EL-4 cells, EL-4 cells treated with 50 μ M NLVS, or adapted EL-4 cells. NLVS-treated cells were pretreated with 50 μ M NLVS for 16 h before the pulse chase⁴. Stabilization was assayed as described²⁹, using the OVA peptide SIINFEKL. Class I molecules were isolated by immunoprecipitation using the Y3 monoclonal antibody or p8 antiserum. Proteins were separated by SDS-PAGE and visualized by fluorography.

Gel filtration of subcellular fractions. Lysates were prepared from control EL-4 and EL-4 adapted cells ($2-3 \times 10^6$ cells) and fractionated by differential centrifugation. The 5-h 100,000g pellets were resuspended in 0.5 ml homogenization buffer (50 mM Tris, pH 7.0, 20% glycerol) and injected into a Superose-6 column (1.5 cm $\times$ 30 cm) at room temperature. The sample was eluted at a flow rate of 0.2 ml min⁻¹. Fractions of 0.5 ml were collected and placed on ice. Aliquots of each fraction (20 μ l) were used to measure hydrolysis of Suc-LLV-MCA and AAF-MCA.

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- Etlinger, J. D. & Goldberg, A. L. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl Acad. Sci. USA* **74**, 54–58 (1977).
- Palombella, V. J., Rando, O. J., Goldberg, A. L. & Maniatis, T. The ubiquitin-proteasome pathway is required for processing the NF- κ B1 precursor protein and the activation of NF- κ B. *Cell* **78**, 773–785 (1994).
- Glötzter, M., Murray, A. W. & Kirschner, M. W. Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132–138 (1991).
- Rock, K. L. *et al.* Inhibition of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761–771 (1994).
- Coux, O., Tanaka, K. & Goldberg, A. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**, 801–847 (1996).
- Seemüller, E. *et al.* The proteasome from *Thermoplasma acidophilum*: A threonine protease. *Science* **268**, 579–582 (1995).
- Löwe, J. *et al.* Crystal structure of the 20S proteasome from the Archaeon *T. acidophilum* at 3.4 Å resolution. *Science* **268**, 533–539 (1995).
- Omura, S. *et al.* Lactacystin, a novel microbial metabolite, induces neurogenesis of neuroblastoma cells. *J. Antibiot.* **44**, 113–116 (1991).
- Fenteany, G. *et al.* Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–730 (1995).
- Dick, L. R. *et al.* Mechanistic studies on the inactivation of the proteasome by lactacystin. *J. Biol. Chem.* **271**, 7273–7276 (1996).
- Groll, M. *et al.* Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463–471 (1997).
- Bogoy, M. *et al.* Covalent modification of the active site Thr of proteasomal β -subunits and the *E. coli* homolog HslV by a new class of inhibitors. *Proc. Natl Acad. Sci. USA* **94**, 6629–6634 (1997).
- Nandi, D., Woodward, E., Ginsburg, D. B. & Monaco, J. J. Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor beta subunits. *EMBO J.* **16**, 5363–5375 (1997).
- Driscoll, J., Brown, M. G., Finley, D. & Monaco, J. J. MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. *Nature* **365**, 262–264 (1993).
- Gaczynska, M., Rock, K. L. & Goldberg, A. γ -Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* **365**, 264–267 (1993).
- Craiu, A. C. *et al.* Lactacystin and clasto-lactacystin β -lactone modify multiple proteasome β subunits and inhibit intracellular protein degradation and MHC class I antigen presentation. *J. Biol. Chem.* **272**, 13437–13445 (1997).
- Bai, A. & Forman, J. The effect of proteasome inhibitor lactacystin on the presentation of transporter associated with antigen processing (TAP)-dependent and TAP-independent peptide epitopes by class I molecules. *J. Immunol.* **159**, 2139–2146 (1997).
- Vinitzky, A. *et al.* The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors: involvement of non-proteasomal cytosolic proteases in antigen processing? *J. Immunol.* **159**, 554–564 (1997).
- Hilt, W. & Wolf, D. H. Proteasomes of the yeast *S. cerevisiae*: genes, structure and function. *Mol. Biol. Rep.* **21**, 3–10 (1995).
- Ghislain, M., Udvardy, A. & Mann, C. *et al.* *S. cerevisiae* 26S protease mutants arrest cell division in G2/metaphase. *Nature* **366**, 358–362 (1993).
- Gordon, C., McGurk, G., Dillon, P., Rosen, C. & Hastie, N. D. Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. *Nature* **366**, 355–357 (1993).
- Tamura, T. *et al.* Tricorn protease—the core of a modular proteolytic system. *Science* **274**, 1385–1389 (1996).
- Kettner, C. & Shaw, E. Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone. *Meth. Enzymol.* **80**, 826–842 (1981).
- Drexler, H. C. A. Activation of the cell death program by inhibition of proteasome function. *Proc. Natl Acad. Sci. USA* **94**, 855–860 (1997).
- Hershko, A. & Ciechanover, A. The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* **61**, 761–807 (1992).
- King, R. W., Jackson, P. K. & Kirschner, M. W. Mitosis in transition. *Cell* **79**, 563–571 (1994).
- Sheaff, R. & Roberts, J. M. End of the line: proteolytic degradation of cyclin-dependent kinase inhibitors. *Chem. Biol.* **3**, 869–873 (1996).
- Hammerling, G. J., Rusch, E., Tada, N., Kimura, S. & Hammerling, U. Localization of allo-determinants on H-2^b determined with monoclonal antibodies and H-2 mutant mice. *Proc. Natl Acad. Sci. USA* **79**, 4737–4741 (1982).
- Ljunggren, H.-G. *et al.* Empty MHC class I molecules come out in the cold. *Nature* **346**, 476–480 (1990).

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Rap1 mediates sustained MAP kinase activation induced by nerve growth factor

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Activation of mitogen-activated protein (MAP) kinase (also known as extracellular-signal-regulated kinase, or ERK)¹ by growth factors can trigger either cell growth or differentiation. The intracellular signals that couple growth factors to MAP kinase may determine the different effects of growth factors: for example, transient activation of MAP kinase by epidermal growth factor stimulates proliferation of PC12 cells¹, whereas they differentiate in response to nerve growth factor, which acts partly by inducing a sustained activation of MAP kinase¹. Here we show that activation of MAP kinase by nerve growth factor involves two distinct pathways: the initial activation of MAP kinase requires the small G protein Ras, but its activation is sustained by the small G protein Rap1. Rap1 is activated by CRK adaptor proteins and the guanine-nucleotide-exchange factor C3G, and forms a stable complex with B-Raf, an activator of MAP kinase. Rap1 is required for at least two indices of neuronal differentiation by nerve growth factor: electrical excitability and the induction of neuron-specific genes. We propose that the activation of Rap1 by C3G represents a common mechanism to induce sustained activation of the MAP kinase cascade in cells that express B-Raf.

PC12 cells are a well studied model of growth-factor specificity. Treatment of PC12 cells with nerve growth factor (NGF) triggers differentiation into sympathetic-like neurons, characterized by electrical excitability, the induction of a set of neuron-specific genes, and neurite outgrowth^{2,3}. The ability of NGF to induce sustained activation of the ERK family of MAP kinases has been implicated in PC12 cell differentiation^{1,4–7}. The molecular mechanisms responsible for sustaining ERK activation are not known. However, signals that limit ERK activation have been identified. In some cells, ERK directs the phosphorylation of the Ras guanine-nucleotide-exchange factor, Sos, to terminate Ras-dependent ERK activation⁸. Therefore, Ras-independent pathways may be required to maintain ERK activation for sustained periods. As Rap1 can also stimulate ERK in PC12 cells⁹, we examined whether Rap1 contributes to NGF action in PC12 cells.

Using an interfering mutant of Rap1, RapN17 (ref. 9), we showed that Rap1 is required for maximal activation of ERK by NGF in PC12 cells. RapN17 blocked the ability of NGF to stimulate the sustained phase of ERK activation in these cells, without inhibiting the initial rapid phase of ERK activation. In contrast, RasN17, a dominant-negative mutant of Ras, blocked only the initial phase of ERK activation by NGF (Fig. 1a). It has been suggested that Ras is essential for NGF signalling to ERKs^{10,11}, but these studies investigated only early time points of stimulation by NGF or used stably transfected clonal variants of PC12 cells rather than wild-type cells. Our results indicate that Ras and Rap1 mediate the initial and the sustained phases of NGF-induced activation of ERK, respectively, and that Ras and Rap1 act largely independently of each other.

As one of the nuclear targets of ERK is Elk-1, a transcription factor of the Ets family⁹, NGF-induced activation of ERK can be monitored by measuring the rate of Elk-1-dependent transcription⁹. In PC12 cells, NGF stimulated Elk-1 more than did EGF (Fig. 1b). Expression of RapN17 reduced NGF-induced activation of Elk-1 to levels observed following stimulation by epidermal

growth factor (EGF), whereas activation of Elk-1 by EGF was unaffected (Fig. 1b)⁹. RasN17 blocked activation of Elk-1 by both agents, indicating that Rap1 and Ras could both be required for the marked activation of Elk-1 stimulated by NGF.

Like other small GTP-binding proteins, Rap1 is active in the GTP-bound state⁹. Both the cyclic AMP analogue 8-(4-chlorophenylthio)-cAMP (8-CPT) and NGF were able to activate Rap1 for prolonged periods (30–45 min) (Fig. 2a). Neither RapN17 nor RasN17 blocked NGF's activation of Ras and Rap1, respectively, indicating that Ras and Rap are activated by parallel pathways (Fig. 2a, b). Rap1 is a selective activator of B-Raf in PC12 cells⁹. When Rap1 was activated by NGF or by 8-CPT, B-Raf was recruited to the membrane, where it formed a stable complex with Rap1 (Fig. 2c). No Rap-associated B-Raf was detected in untreated cells (data not

shown)⁹. The activation of B-Raf by NGF through Rap1 might explain why B-Raf, and not Raf-1, is the principal Raf isoform activated by NGF in PC12 cells¹².

Although constitutive activation of Rap1 is sufficient to trigger neurite outgrowth⁹, it is not necessary for this response: the ability of NGF to induce neurites in PC12 cells was not inhibited by RapN17 at either high (100 ng ml⁻¹)⁹ or low doses (10 ng ml⁻¹) of NGF (data not shown). These results, together with the data from Fig. 1a, demonstrate that neurite outgrowth by NGF does not require sustained activation of ERK. Because transient activation of ERK is not sufficient for neurite outgrowth in the absence of additional signals¹³, our results indicate that morphological differentiation of PC12 cells involves the activation of multiple pathways by NGF.

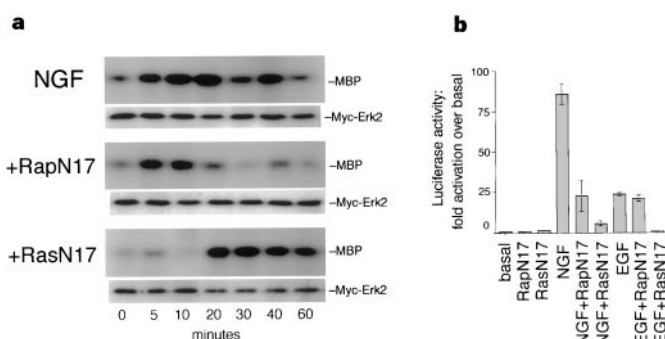


Figure 1 Ras- and Rap-dependent components of NGF-induced activation of ERK. **a**, Stimulation of ERK activity by NGF and inhibition by RapN17 and RasN17. Cells were transfected with Myc-Erk2 alone (top panel), or with RapN17 or RasN17, treated with NGF as indicated and assayed for Myc-Erk2 activity. The position of the Erk2 substrate, myelin basic protein (MBP), is shown. A western blot showing levels of Myc-Erk2 expression is shown below each assay. **b**, Requirement of Rap and Ras for activation of Erk-dependent transcription by NGF. PC12 cells were transfected with Elk-1/Gal4, 5 × Gal4-E1b/luciferase and RapN17 or RasN17, treated with NGF or EGF, and the luciferase activity measured. Standard error is shown ($n = 3$).

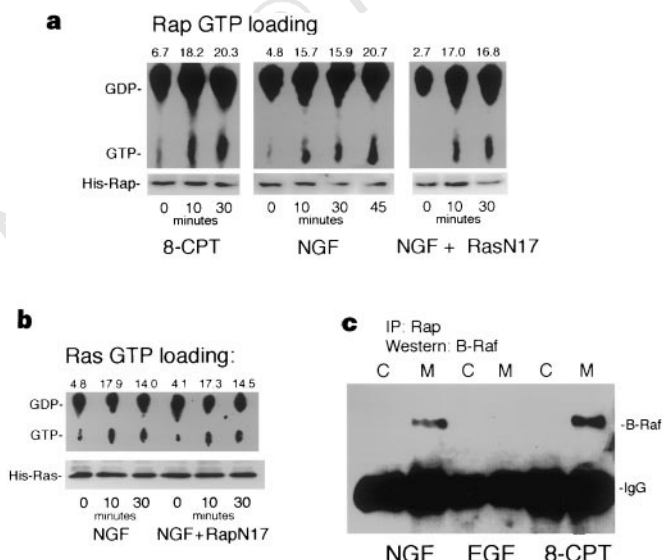


Figure 2 Rap1 activation and association with B-Raf. **a**, Ras-independent activation of Rap1 by 8-CPT and NGF. Rap GTP loading was assayed in NGF or 8-CPT-treated PC12 cells in the presence or absence of RasN17, as indicated. The ratio of GTP/GDP loading is indicated above each lane. **b**, RapN17 does not block Ras activation by NGF. Ras GTP loading was assayed in PC12 cells in the presence or absence of RapN17. In **a** and **b**, the amount of His-Rap and His-Ras proteins in parallel eluates was determined by western blotting (lower panels). **c**, NGF and 8-CPT induce association of Rap1 with B-Raf. Membranes (M) and cytosolic (C) fractions were prepared from PC12 cells treated as indicated. Rap1 immunoprecipitates were examined by western blot using B-Raf antiserum.

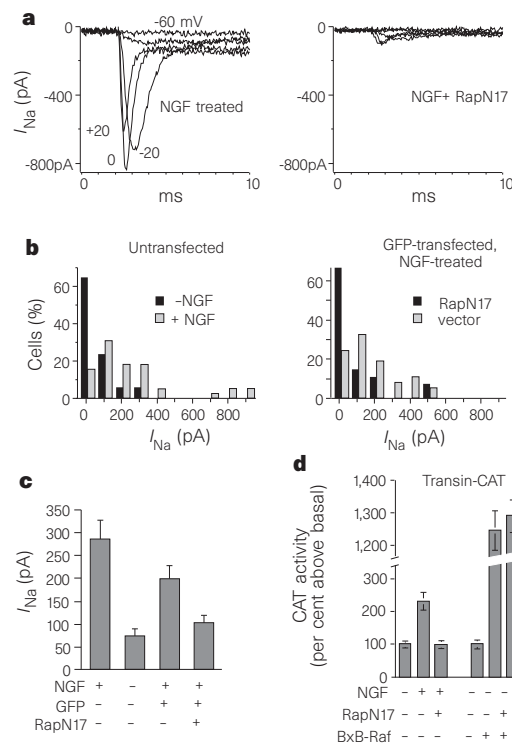


Figure 3 The effect of RapN17 on neuronal differentiation. **a**, Na⁺ currents from PC12 cells after 2–3 days in NGF (10 ng ml⁻¹). Currents from a representative untransfected (left) and RapN17/GFP-transfected cell (right). **b**, Histograms of peak Na⁺ current amplitudes. Left, peak currents from untreated PC12 cells (black; $n = 17$) and PC12 cells treated with NGF (white; $n = 39$). Right, NGF-treated PC12 cells transfected with GFP (2 μ g) plus RapN17 (black; $n = 27$) or GFP alone (white; $n = 37$). **c**, Mean current expressed in pA for each treatment group $\pm$ standard error. **d**, Inhibition of NGF induction of transin by RapN17. PC12 cells were transfected with transin-CAT, RapN17 or BxRaf and treated with NGF.

Sodium currents were induced by NGF in untransfected PC12 cells, as previously reported^{2,14}, and in cells expressing green fluorescent protein (pEGFP-C1) as a marker for transfected cells (Fig. 3a). However, following co-transfection of RapN17, the percentage of GFP-positive cells displaying a significant sodium current was markedly reduced (Fig. 3a, b; right panels). The average peak sodium currents are shown in Fig. 3c. These results indicate that NGF requires Rap1, but not Ras^{15,16}, to induce sodium currents in PC12 cells.

The transcriptional activation of transin (stromelysin) is a marker for neuronal differentiation of PC12 cells by NGF^{16,17}. A reporter plasmid containing the chloramphenicol acetyltransferase (CAT) gene linked to a fragment of the transin promoter (transin-CAT) confers responsiveness to NGF, but not to EGF, in PC12 cells¹⁷. RapN17 blocked the induction of transin-CAT activity by NGF in these cells (Fig. 3d). Therefore, transin, like Elk-1, requires both Ras¹⁶ and Rap1 for full induction by NGF. This might explain why neither EGF (a Ras activator) nor cAMP (a Rap1 activator) is able to induce transin expression, but when applied together they are potent inducers⁷. RapN17 did not block the induction of transin by BxRaf, a constitutively active mutant of Raf-1, suggesting that Rap1 acts upstream or independently of Raf-1 to regulate transin expression.

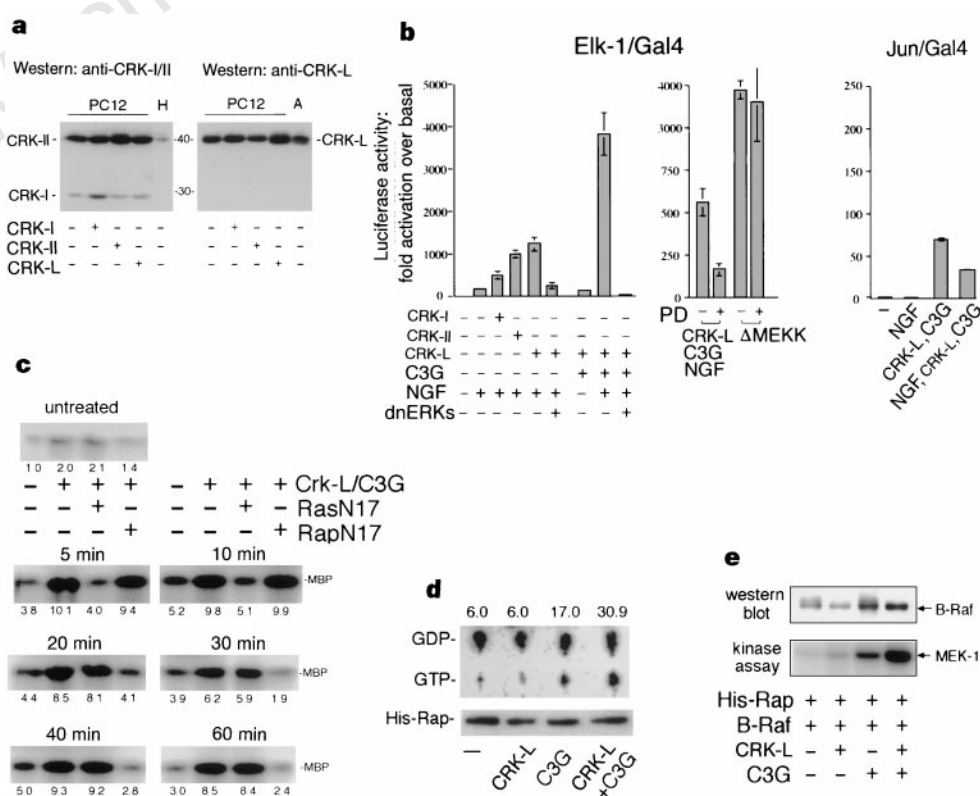
Rap1 activation by NGF might involve the guanine-nucleotide-exchange factor, C3G (refs 18, 19). C3G is a selective activator of Rap1 (ref. 20) and was identified because of its ability to interact with members of the family of CRK adaptor proteins, including Crk-I, Crk-II and Crk-L. *In vivo*, C3G binds specifically to the N-terminal SH3 domain of CRK proteins but not to other SH3-containing proteins such as Grb2 (refs 19, 21–24), indicating that C3G is specifically activated by CRK-dependent pathways. Crk-L is the predominant CRK isoform that interacts with C3G in several

cell types^{23–25}; it is abundant in PC12 cells (Fig. 4a). PC12 cells also express high levels of Crk-II and low, but detectable, levels of Crk-I (Fig. 4a). Activation of Elk-1 by NGF was potently increased by co-transfection of exogenous Crk-II and Crk-L, but only weakly by Crk-I (Fig. 4b, left). In the absence of NGF, the expression of CRK isoforms activated Elk-1 minimally (data not shown). C3G markedly increased activation of Elk-1 by NGF when co-transfected with Crk-L (Fig. 4b), but had only a moderate effect on its own (data not shown).

As Crk-L/C3G also activates the *c-jun* N-terminal kinase JNK²⁶, we used the Jun/Gal4 and Gal4/luciferase reporter system to test whether JNK contributed to the activation of Elk-1 by Crk-L/C3G in PC12 cells²⁷. We found that Crk-L/C3G expression, but not NGF stimulation, increased luciferase activity in this assay (Fig. 4b, right) and that NGF blocked activation of JNK by Crk-L/C3G, indicating that the potentiation of NGF-induced activation of Elk-1 by Crk-L/C3G was not mediated by JNK. Elk-1 activation by NGF/Crk-L/C3G was blocked by interfering mutants of Erk1 and Erk2 (dnERKs) (Fig. 4b, left), and by the MEK inhibitor PD98059 (Fig. 4b, middle). The action of PD98059 was specific: it had no effect on the activation of Elk-1 by a constitutively activated MEK kinase (Δ MEKK). These results demonstrate that NGF potentiates Crk-L/C3G signalling to Elk-1 by activating ERK. This potentiation was demonstrated by using Myc-Erk2 (Fig. 4c): expression of co-transfected Crk-L and C3G increased NGF-induced activation of Myc-Erk2 at all time points. RapN17 blocked the activation of Myc-Erk2 by NGF/Crk-L/C3G only at later times (20, 30, 40 and 60 min) (Fig. 4c). In contrast, RasN17 blocked Erk2 activation at early times (5 and 10 min). As Sos, but not C3G, can activate Ras²⁰, RasN17 may be blocking Crk-L signalling to Sos rather than to C3G at these early times. These results confirm that Ras is required only

Figure 4 Enhancement of NGF signalling by Crk-L and C3G.

a, Expression of CRK isoforms in PC12 cells. PC12 cells were transfected with Crk-I, Crk-II or Crk-L, as indicated, and lysates (20 μ g of protein) were examined by western blot. Left, PC12 cells and control Hela cells (H) were probed with an antibody recognizing both Crk-I and Crk-II (anti-Crk-I/II). Right, PC12 cells and control A-431 cells (A) were probed with anti-Crk-L. **b**, Crk-L/C3G mediate Elk-1 activation by NGF. Elk-1/Gal4 (left and middle panels) and *c-Jun*/Gal4 (right) activities were measured using luciferase assays in cells transfected and treated as indicated. Middle panel: cells were treated with PD98059 (PD) (20 μ M) for 10 min before NGF treatment. **c**, Rap1 mediates Erk2 activation by NGF/Crk-L/C3G. PC12 cells were transfected with the indicated plasmids, and treated with NGF for the indicated times. Relative Myc-Erk2 activities of the transfectants are shown below the corresponding band of phosphorylated MBP. **d**, Activation of Rap1 by Crk-L/C3G. GTP loading was measured in PC12 cells expressing His-Rap and C3G and Crk-L as indicated. **e**, Crk-L and C3G induce the association of B-Raf protein and kinase activity with Rap1. COS-7 cells were transfected as indicated. Top, western blot of B-Raf eluting with His-Rap. Bottom, B-Raf immune complex kinase assays of His-Rap eluates using recombinant MEK-1 as substrate.



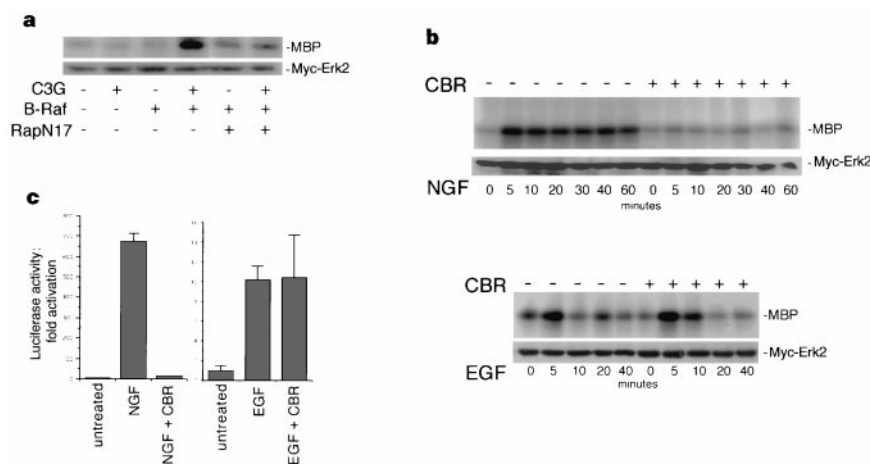


Figure 5 Requirement of Crk/C3G for maximal activation of ERK. **a**, C3G activation of Erk2 requires B-Raf and Rap1. NIH3T3 cells were transfected as indicated and assayed for Myc-Erk2 activity. **b**, CBR inhibits activation of Myc-Erk2 by NGF, but not EGF. PC12 cells were transfected, and treated with NGF (top) or EGF (bottom). The expression of equal amounts of Myc-Erk2 protein in **a** and **b** was confirmed by western blotting (lower panels). **c**, CBR inhibits Elk-1 activation by NGF, but not EGF. Transfected PC12 cells were treated with NGF or EGF and luciferase activity was assayed.

for the early component and Rap1 is required only for the late component of NGF-induced activation of ERK.

C3G stimulated the GTP loading of Rap1 and induced the association of Rap1 with B-Raf, as detected by western blotting and kinase assay (Fig. 4d, e). Both effects of C3G were potentiated by Crk-L (Fig. 4d, e). In NIH3T3 cells, Rap1 activation of ERK requires exogenous B-Raf⁹. The ability of C3G to activate ERK in these cells also required exogenous B-Raf and was blocked by expression of RapN17 (Fig. 5a), demonstrating that C3G activates ERK in a Rap1- and B-Raf-dependent manner.

NGF may use CRK isoforms to activate Ras as well^{21,28}, because Ras activation by NGF can be inhibited by interfering mutants of CRK²¹. These mutants of CRK have no effect on EGF-stimulated ERK activity²⁹, indicating that the ability of CRK proteins to mediate ERK activation is specific for NGF. To test this, we used a truncated mutant of C3G (CBR) containing the CRK-binding region which interferes with CRK function²⁰. When expressed in PC12 cells, CBR inhibited the activation of ERK by NGF at all times but had no effect on EGF signalling to ERK (Fig. 5b). These results indicate that CBR interferes selectively with CRK pathways to block both C3G- and Sos-dependent signals, and confirms that EGF signalling to ERK does not require CRK²⁹. The selective action of CBR on NGF signalling was also reflected by the activation of Elk-1 (Fig. 5c). Therefore, NGF signalling to both Ras and Rap1 may require CRK isoforms.

Crk-L and C3G are activated by several growth factors in many cell types, where they are thought to activate Rap1 (refs 22–25, 30). As Rap1 activates B-Raf, but inhibits Raf-1 (ref. 9), Rap1 could have two opposing functions: to limit ERK activation in B-Raf-negative cells and to increase ERK activation in B-Raf-positive cells. In PC12 cells, and possibly other neuronal cells that express B-Raf, NGF-induced activation of Rap1 promotes a sustained activation of ERK and is required for the induction of electrical excitability and a subset of neuron-specific genes. Furthermore, as B-Raf can convert Rap1 into a positive regulator of ERK, the regulation of B-Raf expression in tissues may provide a new mechanism for modulating growth factor signalling by Rap1. □

Methods

Materials. Polyclonal anti-Rap1 and anti-B-Raf were purchased from Santa Cruz Biotechnology and were used in unbound form for immunoblotting and bound to protein A-Sepharose for immunoprecipitation. The CRK antibody (Transduction Labs) recognizes both Crk-I and II (anti-Crk-I/II). Crk-L antisera and monoclonal antibodies against Ras were from Santa Cruz Biotechnology. Agarose-conjugated monoclonal anti-c-Myc antibody (9E10; Santa Cruz Biotechnology) was used to immunoprecipitate exogenous Myc-Erk2 fusion protein. Nickel-agarose (Ni-NTA-Agarose) was purchased from Qiagen; radioisotopes were from NEN-DuPont; PD98059 was from CalBiochem; all other reagents were from Sigma.

Cell culture. PC12-GR5, COS-7, and NIH3T3 cells were maintained as described⁹. For immune complex assay and western blotting, cells were deprived of serum and maintained in DMEM for 8–16 h at 37°C in 5% CO₂ before treatment with NGF (50 ng ml⁻¹), EGF (50 ng ml⁻¹) or 8-CPT (175 μM), unless otherwise indicated. Cell fractionation and co-precipitation were done as described⁹.

Plasmids and transfection-based assays. Cell lines were grown to ~50% confluence on 100-mm plates that were uncoated (NIH3T3, COS-7 cells) or coated with polylysine (PC12-GR5) before transfection. Transfections were done by using calcium phosphate and following the manufacturer's instructions (GIBCO-BRL). In all experiments, the amount of total DNA transfected was kept constant by addition of pcDNA3 vector (Invitrogen).

For ERK assay, cells were transfected with Myc-Erk2 (5 μg) and assayed for Erk2 activity as described⁹. For B-Raf assay, eluates were immunoprecipitated with B-Raf antiserum and assayed *in vitro* as described⁹, using recombinant MEK-1 as substrate. For luciferase assay⁷, the plasmids encoding Elk-1/Gal4 (3 μg) or c-Jun/Gal4 (3 μg), and 5 × Gal4-E1b/luciferase (3 μg) were transfected into PC12 cells with additional plasmids as indicated. For CAT assay, the plasmid encoding transin-CAT (5 μg) was transfected into PC12 cells together with other plasmids as indicated and assayed as described⁷. Additional cDNAs included His-Rap (15 μg); His-Ras (15 μg); RapN17 (10 μg); RasN17 (10 μg); Crk-I (10 μg); Crk-II (10 μg); Crk-L (10 μg); C3G (10 μg); B-Raf (12 μg); Bx-B-Raf (5 μg); dnERKs (10 μg); ΔMEKK (1 μg); and pEGFP-C1 (2 μg). Elk-1/Gal4, and 5 × Gal4-E1b/luciferase were gifts from R. Maurer; c-Jun/Gal4 was from R. Goodman; cDNA encoding GFP (pEGFP-C1) was from Clontech; transin-CAT was provided by G. Ciment, RasN17 by N. Nathanson, Bx-B-Raf by U. Rapp, Myc-Erk2 by C. Marshall, dnERKs by M. Cobb, ΔMEKK by G. Johnson, C3G by M. Matsuda; Crk-I, Crk-II and Crk-L were provided by B. Druker.

CBR was made by PCR amplification of the cDNA encoding amino acids 279–660 of C3G using oligonucleotide primers containing a *Hind*III site at the 5' end (sense oligonucleotide: 5'-GCGAAGCTTGAGACCATGGATAA-TAGTCTCCACCA-3') and an *Xho*I site at the 3' end (antisense oligonucleotide: 5'-ATTCTCGAGCTGAGCCGACTCAGAGC-3'), and subcloned into pcDNA3. cDNA encoding His-Ras was amplified by PCR from cDNA encoding wild-type Ha-Ras, using specific primers that directed the fusion of the coding region (minus the start methionine) in frame with the polyhistidine amino terminus⁹. All plasmid constructions were sequenced before use.

Electrophysiological studies. PC12 cells were plated on collagen, transfected, and treated with NGF (10 ng ml⁻¹). Two to three days later, whole-cell sodium currents were recorded with a patch-clamp amplifier using 2.5 MΩ pipettes, holding potential -70 mV. The pipette solution contained 140 mM N-methyl glucamine, 10 mM EGTA, 10 mM HEPES, and was adjusted to pH 7.2 with HCl. The external solution contained 140 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 2 mM MnCl₂, 10 mM HEPES, and was adjusted to pH 7.2 with NaOH. Voltage steps were made from -60 mV to +20 mV in 20-mV increments; peak sodium current was measured at 0 mV. Histograms were created from peak currents binned in 100-pA increments and expressed as percentage of cells within each group. All cells used in histograms had input resistances greater than 300 MΩ, confirming that cell membranes were intact.

GTP loading. Cells were transfected with 15 μ g His-Rap or His-Ras and the indicated plasmids as described. Before treatment, cells were serum-starved in DMEM for 4 hours, washed three times and incubated in phosphate-free DMEM at 37 °C. After one hour, 0.5 mCi ml⁻¹ [³²P]orthophosphate in DMEM was added and cells were incubated for an additional 1–2 h. Rap1 was precipitated with Ni-NTA agarose and GTP loading was calculated as described⁹.

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- Marshall, C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179–185 (1995).
- Dichter, M. A., Tischler, A. S. & Greene, L. A. Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature* **268**, 501–504 (1977).
- Greenberg, M. E., Greene, L. A. & Ziff, E. B. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* **260**, 14101–14110 (1985).
- Cowley, S., Paterson, H., Kemp, P. & Marshall, C. J. Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. *Cell* **77**, 841–852 (1994).
- Traverse, S. et al. EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr. Biol.* **4**, 694–701 (1994).
- Pan, M.-G., Wang, Y.-H., Hirsch, D. D., Lubuda, K. & Stork, P. J. S. The Wnt-1 proto-oncogene regulates MAP kinase activation by multiple growth factors in PC12 cells. *Oncogene* **11**, 2005–2012 (1995).
- Yao, H. et al. Cyclic adenosine monophosphate can convert epidermal growth factor into a differentiating factor in neuronal cells. *J. Biol. Chem.* **270**, 20748–20753 (1995).
- Porfiri, E. & McCormick, F. Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor SOS1. *J. Biol. Chem.* **271**, 5871–5877 (1996).
- Vossler, M. et al. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89**, 73–82 (1997).
- Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. & Brugge, J. S. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **78**, 1031–1040 (1992).
- Wood, K. W., Sarnecki, C., Roberts, T. M. & Blenis, J. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, raf-1, and RSK. *Cell* **68**, 1041–1050 (1992).
- Jaiswal, R. K., Weissinger, E., Kolch, W. & Landreth, G. E. Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signaling complex containing B-Raf and HSP90. *J. Biol. Chem.* **271**, 23626–23629 (1996).
- Ihara, S. et al. Dual control of neurite outgrowth by STAT3 and MAP kinase in PC12 cells stimulated with interleukin-6. *EMBO J.* **16**, 5345–5352 (1997).
- Mandel, G., Cooperman, S. S., Maue, R. A., Goodman, R. H. & Brehn, P. Selective induction of brain type II Na⁺ channels by nerve growth factor. *Proc. Natl Acad. Sci.* **85**, 924–928 (1988).
- Fanger, G. R., Erhardt, P., Cooper, G. M. & Maue, R. A. Ras-independent induction of rat brain type II sodium channel expression in nerve growth factor-treated PC12 cells. *J. Neurochem.* **61**, 1977–1980 (1993).
- D'Arcangelo, G. & Halegoua, S. A branched signaling pathway for nerve growth factor is revealed by src-, ras-, and raf-mediated gene inductions. *Mol. Cell. Biol.* **13**, 3146–3155 (1993).
- deSouza, S., Lochner, J., Machida, C. M., Matrisian, L. M. & Ciment, G. A novel NGF-responsive element in the stromelysin-1 (transin) gene that is necessary and sufficient for gene expression in PC12 cells. *J. Biol. Chem.* **270**, 9106–9114 (1995).
- Tanaka, S. et al. C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of Crk and GRB2/ASH proteins. *Proc. Natl Acad. Sci. USA* **91**, 3443–3447 (1994).
- Knudsen, B. S., Feller, S. M. & Hanafusa, H. Four proline-rich sequences of the guanine-nucleotide exchange factor C3G bind with unique specificity to the first Src homology 3 domain of Crk. *J. Biol. Chem.* **269**, 32781–32787 (1994).
- Gotoh, T. et al. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. *Mol. Cell. Biol.* **15**, 6746–6753 (1995).
- Matsuda, M. et al. Crk protein binds to two guanine nucleotide-releasing proteins for the Ras family and modulates nerve growth factor-induced activation of Ras in PC12 cells. *Mol. Cell. Biol.* **14**, 5495–5500 (1994).
- Feller, S. M., Knudsen, B. & Hanafusa, H. Cellular proteins binding to the first Src homology 3 (SH3) domain of the proto-oncogene product c-Crk indicate Crk-specific signaling pathways. *Oncogene* **10**, 1465–1473 (1995).
- Reedquist, K. A. et al. Stimulation through the T cell receptor induces Cbl association with Crk proteins and the guanine nucleotide exchange protein C3G. *J. Biol. Chem.* **271**, 8435–8442 (1996).
- Smit, L., van der Horst, G. & Borst, J. S. Vav, and C3G participate in B cell receptor-induced signaling pathways and differentially associate with Shc-Grb2, Crk, and Crk-L adaptors. *J. Biol. Chem.* **271**, 8564–8569 (1996).
- Uemura, N. et al. The BCR/ABL oncogene alters interaction of the adapter proteins Crk-L and Crk with cellular proteins. *Leukemia* **11**, 376–385 (1997).
- Tanaka, S., Ouchi, T. & Hanafusa, H. Downstream of Crk adaptor signaling pathway: activation of jun kinase by v-Crk through the guanine nucleotide exchange protein C3G. *Proc. Natl Acad. Sci. USA* **94**, 2356–2361 (1996).
- Hirsch, D. D. & Stork, P. J. S. MAP kinase phosphatases inactivate stress-activated protein kinase pathways *in vivo*. *J. Biol. Chem.* **272**, 4568–4575 (1997).
- Tanaka, S. et al. Both the SH2 and SH3 domains of human Crk protein are required for neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* **13**, 4409–4415 (1993).
- Tanaka, M., Gupta, R. & Mayer, B. J. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adaptor proteins. *Mol. Cell. Biol.* **15**, 6829–6837 (1995).
- Boussiotis, V. A., Freeman, G. J., Berezovshaya, A., Barber, D. L. & Nadler, L. M. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* **278**, 124–128 (1997).

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The β_2 -adrenergic receptor interacts with the Na⁺/H⁺-exchanger regulatory factor to control Na⁺/H⁺ exchange

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Stimulation of β_2 -adrenergic receptors on the cell surface by adrenaline or noradrenaline leads to alterations in the metabolism, excitability, differentiation and growth of many cell types. These effects have traditionally been thought to be mediated exclusively by receptor activation of intracellular G proteins¹. However, certain physiological effects of β_2 -adrenergic receptor stimulation, notably the regulation of cellular pH by modulation of Na⁺/H⁺ exchanger (NHE) function, do not seem to be entirely dependent on G-protein activation^{2–7}. We report here a direct agonist-promoted association of the β_2 -adrenergic receptor with the Na⁺/H⁺ exchanger regulatory factor (NHERF), a protein that regulates the activity of the Na⁺/H⁺ exchanger type 3 (NHE3)⁸. NHERF binds to the β_2 -adrenergic receptor by means of a PDZ-domain-mediated interaction with the last few residues of the carboxy-terminal cytoplasmic domain of the receptor. Mutation of the final residue of the β_2 -adrenergic receptor from leucine to alanine abolishes the receptor's interaction with NHERF and also markedly alters β_2 -adrenergic receptor regulation of NHE3 in cells without altering receptor-mediated activation of adenylyl cyclase. Our findings indicate that agonist-dependent β_2 -adrenergic receptor binding of NHERF plays a role in β_2 -adrenergic receptor-mediated regulation of Na⁺/H⁺ exchange.

β_2 -Adrenergic receptors belong to the class of seven-transmembrane-domain receptors for hormones and neurotransmitters that possess extracellular amino termini and intracellular carboxy termini¹. To identify proteins that might interact with the intracellular C-terminal tail of β_2 -adrenergic receptors, we probed tissue extracts from various organs in blot overlay experiments using the β_2 -receptor tail expressed as a fusion protein with glutathione-S-transferase (GST). These overlay assays revealed a single prominent band corresponding to a relative molecular mass of ~50K which was greatly enriched in kidney relative to other tissues. This protein was purified in one step from CHAPS-solubilized bovine kidney extract using a matrix consisting of the C-terminal tail of the β_2 receptor expressed as a GST-fusion protein and adsorbed to glutathione-agarose beads (Fig. 1a). Tryptic digestion and sequencing of the purified peptides yielded two fragments (VGQYIRLVPGSPAEEK

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and LLVVDRETDEFFK); a database search revealed that both fragments matched internal sequence stretches of NHERF, a 50K protein that is expressed in many tissues and at especially high levels in the kidney^{8,9}.

The identity of NHERF as the β_2 -adrenergic receptor-interacting species was confirmed in two ways. First, samples that had been purified from kidney with the β_2 -receptor tail fusion protein bead matrix were specifically recognized by anti-NHERF antibodies (Fig. 1b). Second, purified recombinant NHERF bound to the β_2 -receptor tail in blot overlay experiments (Fig. 1c). The NHERF protein has a bipartite structure, with the two halves of the molecule (domains 1 and 2) containing one PDZ domain each. Full-length NHERF and NHERF domain 1 (the N-terminal 151 amino acids) were each recognized by the β_2 -receptor tail probe whereas in overlay assays NHERF domain 2 (the C-terminal 207 amino acids) was not. Because phosphorylation by cyclic AMP-dependent

protein kinase (PKA) is required for NHERF inhibition of NHE3 (ref. 10), we also examined whether PKA phosphorylation of NHERF would affect its binding to the tail of the β_2 receptor in the overlay experiments. No effect of NHERF phosphorylation by PKA was observed on β_2 -receptor tail binding (Fig. 1c), consistent with the observation that the only consensus PKA phosphorylation sites on NHERF are located in domain 2 (ref. 8).

To examine the NHERF-binding determinants on the β_2 receptor, five truncated versions of the β_2 receptor C-terminal tail were expressed as GST fusion proteins and examined for their ability to bind NHERF in the blot overlay assay (Fig. 2a). The three tails that were truncated from the N-terminal side all interacted with NHERF with an affinity equal to that of the wild-type tail, whereas the two tails that were truncated from the C-terminal side did not detectably bind NHERF. As one of the C-terminal truncations was only missing ten amino acids, these results demonstrate that NHERF binds to the extreme C terminus of the β_2 -receptor tail and suggest that this interaction is mediated by the first PDZ domain of NHERF. PDZ domains bind to three- or four-amino-acid stretches of C-terminal sequence on target proteins¹¹. The first PDZ-domain-containing protein for which a preferred target protein C-terminal sequence was characterized was PSD-95, which was found to prefer Val at the C-terminal position and Thr or Ser at the -2 position of binding partners¹². Subsequent analysis of other PDZ-domain proteins has revealed preferences for different C-terminal sequences¹³. To identify the C-terminal residues on the β_2 -receptor tail required for NHERF binding, we sequentially replaced the C-terminal four amino acids of the β_2 -receptor tail with alanine (Fig. 2b). Mutation of Leu to Ala at the terminal position resulted in a complete loss of NHERF binding to the tail. Mutation of Ser to Ala at the -2 position and Asp to Ala at the -3 position resulted in markedly reduced NHERF binding, whereas mutation of Leu to Ala at the -1 position had no effect.

To examine whether NHERF might interact with full-length β_2 receptors *in vivo*, we used immunocytochemistry to analyse HEK-293 cells expressing epitope-tagged versions of NHERF and either the wild-type β_2 receptor or a mutant β_2 receptor (L413A) in which the final residue was changed from leucine to alanine: according to our *in vitro* binding results, this point mutation should abolish the binding of NHERF to the β_2 receptor. Living cells were incubated with antibodies to detect the tagged β_2 receptors and to promote cell-surface receptor crosslinking into large aggregates that would be easily visible. The cells were then fixed, permeabilized and labelled with antibodies to detect NHERF that had been tagged with a different epitope.

As expected, the β_2 receptors aggregated into large patches in all cells and under all conditions as a result of antibody crosslinking (Fig. 3 a, c, e, g). Under control conditions, staining for NHERF was uniform throughout the cytoplasm and cell membrane (Fig. 3, b, f). Following stimulation of wild-type cells transfected with β_2 receptor by the specific β -adrenergic-receptor agonist isoprenaline, however, NHERF staining was notably more patchy (Fig. 3d), with most of these patches co-localizing with β_2 receptor-staining patches. This effect of isoprenaline was blocked by the β_2 -receptor antagonist propranolol (100 μ M) (data not shown). Treatment of the cells with forskolin (10 μ M) had no effect on the subcellular distribution of NHERF (data not shown), indicating that the changes seen upon agonist stimulation were due to receptor activation and not simply to β_2 -receptor-mediated adenylyl cyclase activation. In contrast to the effect of wild-type β_2 -receptor stimulation on NHERF localization, cells transfected with the L413A mutant receptor showed no agonist-induced change in NHERF subcellular distribution after stimulation with isoprenaline (Fig. 3h). The targeting of NHERF to receptor-rich cellular domains following isoprenaline stimulation of wild-type but not L413A mutant β_2 receptors strongly suggests that full-length wild-type β_2 receptors associate with NHERF in cells, that this association depends on agonist activation of the

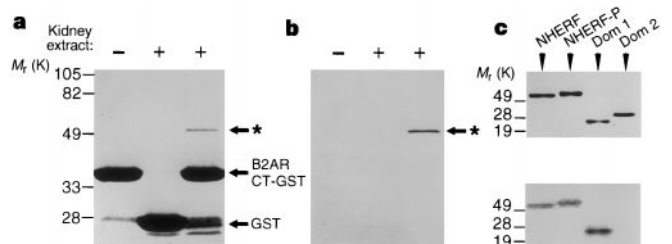


Figure 1 **a**, Purification of a β_2 -receptor tail interacting protein. A Coomassie-blue stained SDS-PAGE gel of proteins eluted from glutathione-agarose resin is shown: from left to right, the resin was bound with β_2 -receptor tail-GST fusion protein alone (B2ARCT-GST), bound with control GST that had been incubated with CHAPS-solubilized bovine kidney extract, or bound with β_2 -receptor tail-GST fusion protein that had been incubated with CHAPS-solubilized bovine kidney extract. A 50K protein (asterisk) from the kidney extract that bound to the β_2 -receptor tail-GST but not to control GST is indicated. **b**, The β_2 -receptor tail interacting protein is recognized by anti-NHERF antibodies. A western blot of the three samples from **a** with an anti-NHERF antibody revealed specific labelling of the purified 50K species (asterisk). **c**, The β_2 -receptor tail binds to recombinant NHERF fusion proteins on a blot overlay. 32 P-phosphorylated β_2 -receptor tail was overlaid onto four blotted samples: NHERF, PKA-phosphorylated NHERF ('NHERF-P'), NHERF domain 1 ('Dom 1') and NHERF domain 2 ('Dom 2'): top, Coomassie-blue stained gel. Results were similar from three experiments.

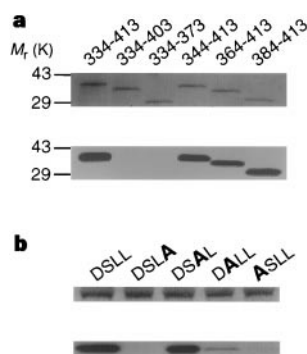


Figure 2 **a**, NHERF binds to the C-terminal 10 amino acids of the β_2 -receptor tail. Five truncated β_2 -receptor tail-GST fusion proteins were expressed and purified, and 5 μ g of each was run on SDS-PAGE (top). A blot of these samples was overlaid with 50 nM NHERF domain-1 fusion protein (bottom). The numbers at the top of the gel represent the amino acids of the wild-type human β_2 receptor to which each truncated mutant fusion protein corresponds. **b**, NHERF binding to the β_2 -receptor tail is inhibited by point mutations at or near the β_2 -receptor C terminus. GST fusion proteins of the β_2 -receptor tail with one of the final four amino acids (DSLL, as labelled at the top of the gel) mutated to alanine were expressed and purified, and 25 μ g of each was run on SDS-PAGE (top). Samples were blotted and overlaid with 50 nM NHERF domain-1 fusion protein (bottom).

receptors, and that it is mediated by the receptors' most C-terminal amino acids.

As NHERF is known to be a regulator of NHE3 (refs 8, 10, 14), we did co-reconstitution experiments to address whether binding of NHERF to the β_2 -receptor tail might affect NHERF regulation of the NHE3. Reconstituted kidney brush-border membranes were prepared¹⁰ in such a way that native NHE3 was fully functional but native NHERF was removed. Recombinant NHERF was then added back to the preparation in the presence of active PKA and ATP, resulting in more than 30% inhibition of NHE3 (Fig. 4). The effect of NHERF on NHE3 function was completely blocked in the presence of the β_2 -receptor tail expressed as a soluble GST fusion protein. The right-hand bar in Fig. 4 indicates that the β_2 -receptor tail fusion protein in the absence of NHERF has no effect on NHE3 function. Further control studies revealed that a GST protein without the β_2 -receptor tail fused to it had no effect on NHERF-mediated inhibition of NHE3 (data not shown). These results indicate that the binding of NHERF to the β_2 -receptor tail prevents NHERF from regulating NHE3, either by preventing the NHERF/NHE3 interaction or by altering the conformation of NHERF so that it can no longer inhibit NHE3.

To test whether binding of NHERF to the β_2 receptor affects adrenergic regulation of NHE3, we examined the effect of β_2 receptor stimulation on NHE3 function in intact cells in a mutant Chinese hamster ovary (CHO) cell line^{15,16} which stably expresses NHE3 but lacks all other NHE isoforms. CHO cells express high levels of NHERF¹⁴, a finding we verified by western blot analysis of our mutant CHO cells (data not shown). CHO cells expressing NHE3 were stably transfected with either wild-type β_2 receptor or the L413A mutant receptor. The two cell lines were similar with respect to ligand binding ($B_{\max}$ for both cell lines was 1.4 pmol receptor per mg total protein) and agonist-induced cAMP accumulation (wild-type line showed $1.28 \pm 0.29\%$ conversion; the L413A mutant line, $1.09 \pm 0.25\%$; non-transfected CHO cells, $0.05 \pm 0.02\%$). Stimulation with forskolin inhibits Na^+/H^+

exchange in CHO cells expressing NHE3 (refs 15, 16), and we found an equivalent amount of forskolin-induced inhibition of Na^+/H^+ exchange in the two stable β_2 receptor cell lines (Fig. 5). The two cell lines differed markedly, however, with regard to adrenergic regulation of Na^+/H^+ exchange: stimulation with isoprenaline of the wild-type β_2 receptor cell line had little effect on NHE3 activity (Fig. 5, left), whereas isoprenaline stimulation of the L413A mutant β_2 receptor cell line inhibited NHE3 activity by $\sim 50\%$ (Fig. 5, right).

The effect of forskolin on NHE3 function is believed to be mediated by PKA phosphorylation of both the NHE3 and NHERF, which leads to NHERF binding to and inhibition of NHE3 (refs 15, 16). According to this model, activation of G_s -protein-coupled receptors such as the β_2 receptor should also inhibit NHE3 activity. Consistent with this, agonist activation of the L413A mutant β_2 receptor does inhibit NHE3, but agonist activation of the wild-type receptor does not. As each receptor activates adenylyl cyclase to the same extent and the only apparent difference between the two receptors is that the wild-type receptor binds NHERF and the mutant does not, the wild-type receptor probably mediates two distinct effects following agonist activation: it binds G_s , activating adenylyl cyclase, but it also binds NHERF, thereby preventing the NHE3 inhibition that would be expected to follow adenylyl cyclase activation.

The effect of hormone stimulation on Na^+/H^+ exchanger activity is not completely understood^{17–19}: for example, β_2 receptor stimulation regulates both NHE1 (refs 2–5) and NHE3 (refs 6, 7) in a manner that cannot be explained by the ability of β_2 receptors to activate adenylyl cyclase through G-protein signalling. These apparently anomalous findings can be explained, at least in part, by the discovery that β_2 receptors bind in an agonist-dependent manner to a protein, NHERF, which is known to inhibit NHE activity. In the case of NHE3, activation of PKA by stimulation with parathyroid hormone or by incubation with cAMP analogues inhibits NHE3 activity in membrane-reconstitution studies^{20–22} and decreases sodium reabsorption in the kidney proximal tubule²³. However, stimulation of β_2 receptors paradoxically increases sodium reabsorption in the kidney proximal tubule by apparently stimulating NHE3 activity^{6,7}. This paradox could be explained if there is a basal level of PKA activation and thus of NHERF-mediated NHE3 inhibition in proximal tubules: the binding of activated β_2 receptors to NHERF might relieve this basal inhibition. Receptor activation may thus stimulate NHE3 activity if the receptor density is high enough to bind a significant fraction of activated cellular NHERF.

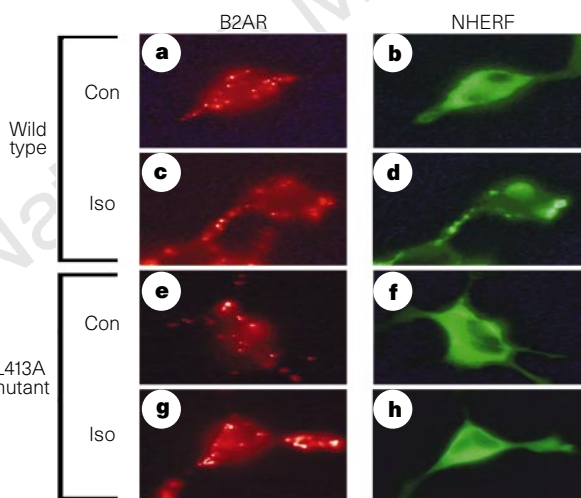


Figure 3 NHERF co-localizes in cells with full-length wild-type β_2 receptor, but not with full-length L413A mutant β_2 receptor, after receptor stimulation with agonist. Left panels show immunostaining of Flag-tagged β_2 receptor (B2AR) over-expressed in HEK-293 cells (red); right panels show HA-tagged NHERF staining in the same cells (green). Cells are shown under control conditions (Con), when β_2 receptor distribution was extremely patchy (a, e) because receptors were crosslinked with antibody (see Methods) and NHERF distribution was quite uniform (b, f). Following stimulation with $50 \mu\text{M}$ isoprenaline (Iso) the pattern of β_2 receptor distribution was unchanged (c), but NHERF staining was more patchy (d), with most of the patches corresponding to regions rich in β_2 receptor staining. This agonist induced co-localization of β_2 receptor and NHERF was not observed in the cells expressing the L413A mutant β_2 receptor (g, h). Cells are representative of 4–6 independent experiments.

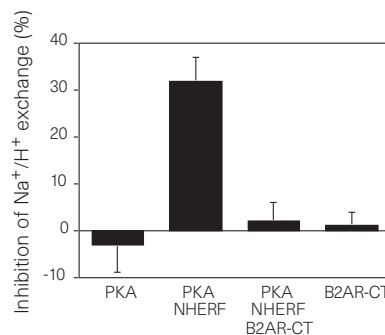


Figure 4 β_2 -receptor tail-GST fusion protein blocks the ability of NHERF to regulate NHE3. The effects of various recombinant proteins on Na^+/H^+ exchange mediated through NHE3 in a partially purified reconstituted brush-border membrane preparation were examined. Addition of PKA (50 U ml^{-1}) had no effect, but addition of NHERF ($1 \mu\text{g ml}^{-1}$) along with PKA gave $>30\%$ inhibition of sodium flux. This effect was blocked by co-application of β_2 -receptor tail-GST fusion protein ($1 \mu\text{g ml}^{-1}$; B2AR-CT); the receptor tail-GST by itself ($1 \mu\text{g ml}^{-1}$) had no effect on sodium flux. Bars and error bars represent the mean $\pm$ s.e.m. for 3 experiments. The average basal value for $^{22}\text{Na}^+$ uptake in these experiments was $15.8 \text{ pmol per mg protein}$.

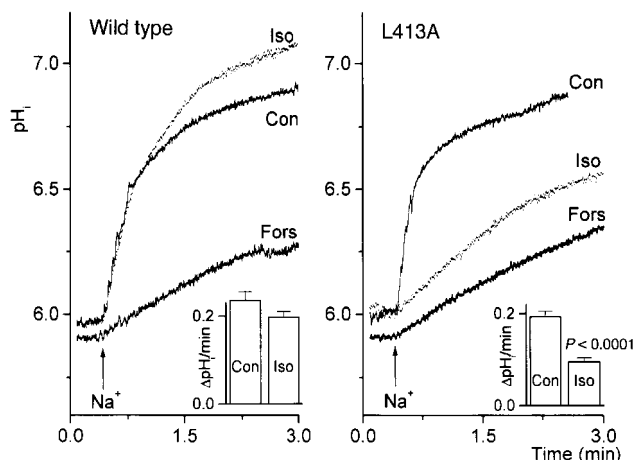


Figure 5 Agonist stimulation of L413A mutant β_2 receptors but not wild-type β_2 receptors inhibits NHE3 in whole cells. pH_i was measured fluorometrically in stable cell lines, expressing either NHE3/wild type β_2 receptor (left) or NHE3/L413A mutant β_2 receptor (right), which were acid-loaded (see Methods) and bathed in Na^+ -free solution. Where indicated, NHE was activated by addition of Na^+ -rich medium (arrow). In the presence of forskolin ('Fors'), NHE3 activity was inhibited in both cell lines. This effect was mimicked by stimulation of the L413A mutant receptor cell line with isoprenaline ('Iso') but not by stimulating the wild-type β_2 -receptor cell line. Traces are representative of at least 4 independent experiments for each condition. Inset, quantification of the initial rate of pH_i recovery after an acute acid load. Values were calculated by deriving the change of pH_i with time every 2.5 s for 60 s after Na^+ addition. Values are means $\pm$ s.e. for 6 and 8 determinations for untreated ('Con') and isoprenaline-treated wild-type β_2 -receptor-expressing cells, respectively, and for 12 and 10 determinations for untreated and isoprenaline-treated L413A-mutant-receptor-expressing cells, respectively. Statistics were done using the Student's *t*-test.

The agonist-dependence of the association between β_2 receptors and NHERF is analogous with the association of other proteins with β_2 receptors: for example, both G proteins and β -arrestins associate with β_2 receptors in an agonist-promoted fashion^{1,24}. However, the primary determinants of G-protein¹ and β -arrestin²⁴ interaction with the β_2 receptor are located in the third intracellular loop of the receptor, whereas NHERF interacts with the receptor C-terminal tail. As mutant β_2 receptors with truncated C-terminal tails signal normally through adenylyl cyclase^{25–27}, it has been assumed that the C-terminal tail is not essential for β_2 receptor signalling. Our results, however, indicate that the β_2 receptor tail is important for signal generation, in particular for the regulation of NHE activity; they also reveal a mechanism distinct from G-protein activation by which seven-transmembrane-domain receptors like the β_2 receptor can influence intracellular events. □

Methods

SDS-PAGE and tissue overlays. A probe was made using β_2 -receptor tail GST fusion protein (the C-terminal 80 amino acids of the human β_2 -adrenergic receptor) adsorbed to glutathione-agarose beads and phosphorylated with $2 \mu\text{g ml}^{-1}$ PKA and 0.1 mCi ml^{-1} [^{32}P]-ATP for 1 h at 30°C . The beads were then washed extensively with PBS and the radiolabelled β_2 -receptor tail was cleaved away from the GST-bead mix using recombinant thrombin in a buffer supplied by the manufacturer (Boehringer Mannheim). Overlay experiments using this probe were done by running samples of bovine tissues ($50 \mu\text{g}$ per lane) on 4–20% SDS-PAGE (Novex) for 1 h at 150 V and then blotting onto nitrocellulose for 40 min at 12 V. Blots were blocked in 2% milk/0.1% Tween-20 in PBS ('blot buffer') and incubated with the phosphorylated β_2 -receptor tail probe in blot buffer for 1 h. Blots were washed five times with blot buffer, once in PBS, and then exposed to Kodak X-OMAT film.

Purification and sequencing. To purify β_2 -receptor-interacting proteins, salt-washed bovine kidney membranes (500 mg total protein) were solubilized for

1 h in a buffer containing 10 mM HEPES, pH 7.5, 50 mM NaCl, 1% CHAPS, 1 mM PMSE, 1 mM benzamide and 1 mM EDTA ('solubilization buffer'). After a 20 min centrifugation at 48,000g, to remove insoluble material, the soluble supernatant was incubated with β_2 -receptor tail-GST fusion protein adsorbed to glutathione-agarose for 1 h at 4°C . The beads were pelleted by brief centrifugation and washed five times with 40 ml fresh solubilization buffer. Finally, the beads were incubated with SDS-PAGE sample buffer for 30 min at 37°C to elute adsorbed proteins from the bead matrix. Samples were then run on 4–20% SDS-PAGE and visualized by staining with Coomassie blue. Peptides for sequencing were generated by cutting the bands of interest out of the gel, followed by trypsin digestion and HPLC purification.

Fusion protein overlays and western blotting. Hexahistidine-tagged and S-tagged NHERF, NHERF domain 1 (1–151) and NHERF domain 2 (152–358) fusion proteins were created by insertion of rabbit NHERF cDNA⁸ into pET-30A (Novagen). Fusion proteins were expressed according to manufacturer's instructions. Purified NHERF fusion proteins were run on SDS-PAGE and overlay assays done as described. In other experiments, wild-type or mutant β_2 -receptor tails expressed as GST fusion proteins were examined for NHERF binding by a far-western blot overlay technique. Mutant β_2 -receptor tail-GST constructs were created by PCR using mutant sequence oligonucleotides and inserting the PCR products in a pGEX-2T vector (Pharmacia); all mutations were confirmed by sequencing with an ABI377 automated sequencer. Fusion proteins were run on SDS-PAGE gel, blotted and overlaid with 50 nM NHERF domain-1 fusion protein in blot buffer for 1 h at room temperature. Blots were washed three times, incubated for 1 h at room temperature with an HRP-conjugated anti-S-tag antibody (Novagen), washed three more times and visualized with an enhanced chemiluminescence kit (Boehringer Mannheim).

An anti-NHERF antibody was created by injecting full-length NHERF fusion protein into rabbits, boosting at four weeks and then bleeding at 10 weeks. The blood serum was passed over a DEAE Affi-gel blue column (BioRad). The resulting purified IgG fraction demonstrated specific binding to recombinant NHERF and NHERF domain 1 but not to other fusion proteins. This antibody also binds to a single major 50K species in bovine kidney (presumably bovine NHERF). Western blots were performed via standard procedures.

Immunocytochemistry. A haemagglutinin (HA)-tagged NHERF construct was prepared by subcloning rabbit full-length NHERF into a modified version of the pBK-CMV vector (Stratagene) which places an HA tag at the N terminus of expressed proteins. HEK-293 cells on coverslips in 6-well plates were co-transfected with $0.5 \mu\text{g}$ of this construct and $0.5 \mu\text{g}$ of a Flag-tagged β_2 -adrenergic receptor construct in the vector pCDNA1 (ref. 28) by calcium phosphate co-precipitation²⁸. Two days later, cells were washed with MEM (Gibco-BRL) and incubated for 30 min at room temperature with M2 monoclonal Flag antibody (Kodak) at a dilution of 1:400. Following three washes with MEM, cells were incubated with a goat anti-mouse Texas red-conjugated whole secondary antibody (Molecular Probes) at a dilution of 1:250 for 30 min. This allows visualization of the transfected β_2 receptors and artificially induces clustering of the receptors into large patches so that the co-localization of proteins with β_2 receptors may be studied²⁹. After three washes with MEM, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 1% BSA/0.04% saponin in PBS ('saponin buffer'), then incubated successively with rabbit anti-HA (Berkeley Antibody) at 1:100 in saponin buffer and a fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes) at 1:250; each step was followed by three washes with 1 ml saponin buffer. Coverslips were examined using a Leica DM model 50 microscope at 100 $\times$ magnification. Control experiments used untransfected or singly transfected cells to verify that each antibody was specifically detecting the appropriate transfected protein.

Creation of wild-type or L413A mutant β_2 receptor stable cell lines. A Flag-tagged L413A mutant β_2 receptor was prepared by PCR amplification using a primer bearing the desired mutation followed by replacement of a cassette in the Flag-tagged wild-type β_2 receptor construct. NHE3-AP1 cells^{15,16} were transfected by the calcium phosphate method²⁸ with the plasmid pSV2-neo and either the Flag-tagged wild-type β_2 receptor or the Flag-tagged L413A mutant receptor. Stable transfectants were selected for two weeks by adding 1 mg ml^{-1} G418 to the medium. Transfection was confirmed by immunocytochemistry, radioligand binding and adenylyl cyclase studies³⁰.

Reconstitution assay. The preparation of kidney brush-border membrane

vesicles for reconstitution assays has been described^{10,20–22}. Briefly, the internal pH of the vesicles was set at 6.0 by dialysis. The final uptake solution contained 1 mM ²²Na⁺, 250 mM mannitol, 30 mM potassium gluconate, 1 μg ml⁻¹ valinomycin and 50 mM Tris/MES at either pH 6.0 or 8.0. Sodium uptake was determined by application of the reaction mix to 1 ml Dowex 50X8 (Tris), 100-mesh columns and rapid elution with vacuum suction with 1 ml 300 mM mannitol (pH 8.0) at 0°C. Under these voltage-clamped conditions, the proton-stimulated component of sodium uptake was taken as a measure of Na⁺/H⁺ exchange rate.

Quantification of Na⁺/H⁺ exchanger activity by cytosolic pH measurement. The intracellular pH (pH_i) of small groups of cells was determined by microphotometry of the fluorescence emission of the pH-sensitive dye, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein, using dual wavelength excitation^{15,16}. Cells grown to 70–80% confluence on glass coverslips were concurrently loaded with 25 mM NH₄Cl and 2 mg ml⁻¹ of the acetoxymethyl ester precursor of 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein in PBS for 10 min at 37°C. Where indicated, 10 μM forskolin or 50 μM isoprenaline was also added. Acid loading was accomplished by washing cells with a Na⁺-free medium containing (in mM): 117 N-methyl-D-glucammonium chloride, 1.66 MgSO₄, 1.36 CaCl₂, 5.36 KCl, 25 HEPES, 5.55 glucose, pH 7.5, 290 ± 10 mosM. Sodium-dependent pH_i recovery was then initiated by perfusing the cells with a Na⁺-rich solution composed of (in mM): 117 NaCl, 1.66 MgSO₄, 1.36 CaCl₂, 5.36 KCl, 25 HEPES, 5.55 glucose, pH 7.5. To measure fluorescence, the coverslip was placed into a holding chamber attached to the stage of a Nikon Diaphot TMD inverted microscope equipped with a Nikon Fluor 40x/1.3 N.A. oil-immersion objective. Clusters of 6–12 cells were selected for analysis with an adjustable diaphragm. Excitation light provided by a Xenon lamp was alternately selected using 495 ± 10 nm and 445 ± 10 nm filters at a rate of 50 Hz and then reflected onto the cells by a 510-nm dichroic mirror. Emitted light was directed to the photometer through a 530 ± 30 nm band-pass filter. Photometric data were acquired at 10 Hz using a 12-bit A/D board (Labmaster, National Instruments) interfaced to a Dell 486 computer and analysed with Felix software (Photon Technologies). Calibration of fluorescence intensity with pH_i was done in the presence of 5 mM nigericin in high-K⁺ medium (140 mM KCl, 20 mM HEPES, 1 mM MgCl₂, 5 mM glucose) as described^{15,16}.

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- Dohlman, H. G., Thorner, J., Caron, M. G. & Lefkowitz, R. J. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**, 653–688 (1991).
- Barber, D. L., McGuire, M. E. & Ganz, M. B. β-Adrenergic and somatostatin receptors regulate Na–H exchange independent of cAMP. *J. Biol. Chem.* **264**, 21038–21042 (1989).
- Ganz, M. B., Pachter, J. A. & Barber, D. L. Multiple receptors coupled to adenylate cyclase regulate Na–H exchange independent of cAMP. *J. Biol. Chem.* **265**, 8989–8992 (1990).
- Barber, D. L. & Ganz, M. B. Guanine nucleotides regulate β-adrenergic activation of Na–H exchange independently of receptor coupling to G_s. *J. Biol. Chem.* **267**, 20607–20612 (1992).
- Barber, D. L., Ganz, M. B., Bongiorno, P. B. & Strader, C. D. Mutant constructs of the β-adrenergic receptor that are uncoupled from adenylyl cyclase retain functional activation of Na–H exchange. *Mol. Pharmacol.* **41**, 1056–1060 (1992).
- Bellow-Reuss, E. Effect of catecholamines on fluid reabsorption by the isolated proximal convoluted tubule. *Am. J. Physiol.* **238**, F347–F352 (1980).
- Weinmann, E. J., Sansom, S. C., Knight, T. F. & Senekjian, H. O. Alpha and beta adrenergic agonists stimulate water absorption in the rat proximal tubule. *J. Membrane Biol.* **69**, 107–111 (1982).
- Weinman, E. J., Steplock, D., Wang, Y. & Shenolikar, S. Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane Na⁺–H⁺ exchanger. *J. Clin. Invest.* **95**, 2143–2149 (1995).
- Reczek, D., Berryman, M. & Bretscher, A. Identification of EBP50: a PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. *J. Cell Biol.* **139**, 169–179 (1997).
- Weinman, E. J., Steplock, D. & Shenolikar, S. cAMP-mediated inhibition of the renal brush border membrane Na⁺–H⁺ exchanger requires a dissociable protein cofactor. *J. Clin. Invest.* **92**, 1781–1786 (1993).
- Sheng, M. PDZs and receptor/channel clustering: rounding up the latest suspects. *Neuron* **17**, 575–578 (1996).
- Kornau, H. C., Schenker, L. T., Kennedy, M. B. & Seeburg, P. H. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**, 1737–1740 (1995).
- Songyang, Z. et al. Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science* **275**, 73–77 (1997).
- Yun, C. H. C. et al. cAMP-mediated inhibition of the epithelial brush border Na⁺/H⁺ exchanger, NHE3, requires an associated regulatory protein. *Proc. Natl Acad. Sci. USA* **94**, 3010–3015 (1997).
- Cabado, A. G. et al. Distinct structural domains confer cAMP sensitivity and ATP dependence to the Na⁺/H⁺ exchanger NHE3 isoform. *J. Biol. Chem.* **271**, 3590–3599 (1996).
- Kurashima, K. et al. Identification of sites required for down-regulation of Na⁺/H⁺ exchanger NHE3 activity by cAMP-dependent protein kinase. Phosphorylation-dependent and -independent mechanisms. *J. Biol. Chem.* **272**, 28672–28675 (1997).
- Weinman, E. J. & Shenolikar, S. Regulation of the renal brush border membrane Na⁺/H⁺ exchanger. *Annu. Rev. Physiol.* **55**, 289–304 (1993).
- Noel, J. & Pouyssegur, J. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na⁺/H⁺ exchanger isoforms. *Am. J. Physiol.* **268**, C283–C296 (1995).
- Orlowski, J. & Grinstein, S. Na⁺/H⁺ exchangers of mammalian cells. *J. Biol. Chem.* **272**, 22373–22376

(1997).

- Kahn, A. M., Dolson, G. M., Hise, M. K., Bennett, S. C. & Weinman, E. J. Parathyroid hormone and dibutyryl cAMP inhibit Na⁺/H⁺ exchange in renal brush border vesicles. *Am. J. Physiol.* **248**, F212–F218 (1985).
- Dolson, G. M., Hise, M. K. & Weinman, E. J. Relationship among parathyroid hormone, cAMP and calcium in proximal tubule sodium transport. *Am. J. Physiol.* **249**, F409–F416 (1985).
- Weinman, E. J., Shenolikar, S. & Kahn, A. M. cAMP-associated inhibition of Na⁺–H⁺ exchanger in rabbit kidney brush-border membranes. *Am. J. Physiol.* **252**, F19–F25 (1987).
- Agus, Z. S., Puschet, J. B., Senesky, D. & Goldberg, M. Mode of action of parathyroid hormone and cyclic adenosine 3'–5' monophosphate on renal-tubular phosphate reabsorption in the dog. *J. Clin. Invest.* **50**, 617–626 (1971).
- Ferguson, S. S. G., Barak, L. S., Zhang, J. & Caron, M. G. G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* **74**, 1095–1110 (1996).
- Strader, C. D. et al. The carboxyl terminus of the hamster β-adrenergic receptor expressed in mouse L cells is not required for receptor sequestration. *Cell* **49**, 855–863 (1987).
- Bouvier, M. et al. Removal of phosphorylation sites from the β₂-adrenergic receptor delays the onset of agonist-promoted desensitization. *Nature* **333**, 370–373 (1988).
- Cheung, A. H., Sigal, I. S., Dixon, R. A. F. & Strader, C. D. Agonist-promoted sequestration of the β₂-adrenergic receptor requires regions involved in functional coupling to G_s. *Mol. Pharmacol.* **35**, 132–138 (1989).
- Opperman, M. et al. Monoclonal antibodies reveal receptor specificity among G protein-coupled receptor kinases. *Proc. Natl Acad. Sci. USA* **93**, 7649–7654 (1996).
- Barak, L. S., Ferguson, S. S. G., Zhang, J. & Caron, M. G. A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J. Biol. Chem.* **272**, 27497–27500 (1997).
- Samama, P., Cotecchia, S., Costa, T. & Lefkowitz, R. J. A mutation-induced activated state of the β₂-adrenergic receptor: extending the ternary complex model. *J. Biol. Chem.* **268**, 4625–4636 (1993).

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corrections

Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein

Stefan Brocke, Koenraad Gijbels, Mark Allegretta, Iris Ferber, Christopher Piercy, Thomas Blankenstein, Roland Martin, Ursula Utz, Nathan Karin, Dennis Mitchell, Timo Veromaa, Ari Waisman, Amitabh Gaur, Paul Conlon, Nicholas Ling, Paul J. Fairchild, David C. Wraith, Anne O'Garra, C. Garrison Fathman & Lawrence Steinman

Nature **379**, 343–346 (1996)

In this Letter, "96P" indicates a proline at residue 96, and not phenylalanine as published. □

A new pattern for helix–turn–helix recognition revealed by the PU.1 ETS-domain–DNA complex

Ramadurgam Kodandapani, Frédéric Pio, Chao-Zhou Ni, Gennaro Piccialli, Michael Klemsz, Scott McKercher, Richard A. Maki & Kathryn R. Ely

Nature **380**, 456–460 (1996)

In the legend to Fig. 3, lines 11 and 12 should read: "Arg 232 (NH1) makes two hydrogen bonds with G9(06) and G9(N7)." In panel b, a dashed line indicating a hydrogen bond to base A10 should be deleted. The coordinates for the complex remain unchanged as originally submitted to the Brookhaven Protein Data Bank under accession number 1 pue. □

Federation special

Focusing on experimental biology, a variety of products for image analysis and microscopy are presented, as well as a line of incubators, a *Saccharomyces pombe*-based protein expression system and new laboratory hardware.

Cellmax

From Cellco

A new catalogue covers the new line of systems and modules for artificial, capillary cell culture

Using hollow-fibre cartridge technology, these systems are designed for the culture of hybridoma cell lines, recombinant CHO and 293 cell lines, endothelial cells under shear stress and lymphocyte expansion. Systems are available for the production of 5–200 mg of monoclonal antibody per harvest and for the culture of up to $5 \times 1,010$ cells, including the new Cellmax BigFoot system for medium- to large-scale cell culture. According to the manufacturer, Cellmax technology provides for medium flow without the use of a peristaltic pump, extending cartridge lifetime for up to six months. Other cell culture applications include cellular co-cultivation, tumour cell lines and cytokine production.

Reader Enquiry No. 100

Focal point

Eclipse E600FN

From Nikon

A new physiology and patch clamp microscope offers a focusing nose piece

With this system, only the nosepiece moves up and down during focusing, providing the space needed for micromanipulations. Designed for electrophysiology studies, this workstation allows objectives to be changed in the close confines of a Petri dish or deep chamber, without disturbing the specimen set up. This microscope combines a two-position focusing nosepiece that ensures the water-dipping objective is correctly positioned in the vessel. The E600FN provides the space needed for at least five micromanipulators and is well suited for work with semi-opaque specimens and tissue cultures. By utilizing the new CFI60 series Fluor W water immersion objectives, a wide 45° angle for micromanipulation has been created. The larger space between the field lens



Nikon's E600FN physiology workstation.

and the condenser makes it easy to add options, such as shutters or filter wheels. A front-mounted lever ensures correct positioning of the water dipping objective in the vessel and eliminates the need to use the focus knob, says Nikon. It also prevents the objective from hitting the deep chamber. The nosepiece focusing system and fixed stage should help to eliminate vibration. Bolt-down brackets can be used to attach the stand, an isolation table or x,y translation assembly. Even the nosepiece and binocular, light-path prism detents can be turned off to eliminate these as sources of vibration.

Reader Enquiry No. 101

CytoGem

From Packard Instruments

A protein fusion and organelle targeting system for intracellular localization

This new system can fuse heterologous proteins to each of three ultrabright CytoGem proteins: Emerald-green (emd), Topaz-gold (tpz) and Sapphire-blue (sph). For each of these, two vectors are available for protein fusion to the N or C terminus of the ultrabright GFP. Fusions are expressed using a cytomegalovirus (CMV) promoter, which is said to allow for very high constitutive expression of protein in a variety of cell types. All six vectors (pGFPemd-N and -C, pGFPtpz-N and -C, and pGFPsph-N and -C) share the same backbone and multiple cloning site. Furthermore, because of the fluorescence spectral properties of Sapphire-blue and Topaz-gold, dual-labelling assays using these two GFPs can be performed. Packard has also introduced a panel of organelle-targeted, Sapphire-blue proteins. Different organelle targeting signals have been subcloned in pGFPsph-N or pGFPsph-C vectors. These organelle targeting sequences guide the Sapphire-blue protein to a single organelle in mammalian cells, including mitochondria, nucleus, peroxisome, cytoplasm, plasma membrane and endoplasmic reticulum.

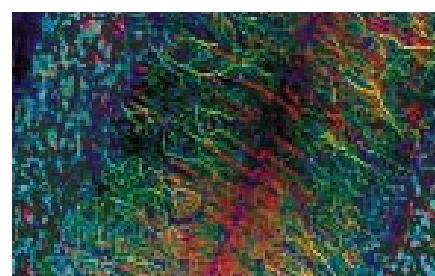
Reader Enquiry No. 102

Landmark

From Bio-Rad

Kits that provide bright three-colour images for labelling a wide range of tissue types

A new method for tissue labelling in confocal microscopy now produces bright, three-colour images, as well as reducing development time. These three kits may also be used for localizing proteins in virtually any type of



Psychedelic neuronal imaging with Landmark.

tissue. The simple methods are said to produce distinct, bright labelling, which is colour balanced for confocal microscopes equipped with Kr/Ar laser lines. The colour clarity is achieved with visualized fluorescent dyes that have wavelengths selected for minimal cross-channel bleeding. The kits can be tailored to individual requirements — scientists can use their own primary antibody with either anti-rabbit or anti-mouse fluorescein-labelled secondary antibodies provided by the manufacturer. Alternatively, a choice of anti-parvalbumin, anti-MAP2, anti-synaptophysin or anti-NFP primary antibodies can be supplied for labelling important landmarks in brain tissue. For either approach, the kits include anti-GFAP primary antibody and an appropriate Cy5-labelled secondary antibody, with a nucleic acid stain, providing a third colour label for cell nuclei.

Reader Enquiry No. 103

LD Achroplan Ph2 objective

From Carl Zeiss

High-magnification, high numerical aperture, long working distance $\times 63$ phase objective for tissue culture vessels

Applications such as micro-dissection or micro-injection, or those where micro-positioning of electrodes is needed, require both high resolution and high magnification to be carried out. The new LD Achroplan $\times 63/10.75$ Korr. Ph2 objective has been created to accommodate this need. A high numerical aperture of 0.75, approximately 0.1 higher than the equivalent $\times 40$ objective, and a correction for window thickness between 0–1.5 mm makes this objective suitable for viewing both through culture dishes and through coverslips. It can also be used for phase-contrast and for bright-field applications, and has a complementary DIC prism for Nomarski differential interference contrast. It is also suitable for fluorescence work even at wavelengths as low as 340 nm.

Reader Enquiry No. 104



Microscopy-Tutor software developed to train would-be microscopists.

Microscopy-Tutor

From Univ. of Washington School of Med.
Microscopy training software for healthcare and laboratory professionals

Published by Lippincott-Raven Publishers, this is an interactive computer program that guides trainees through the basic principles and practice of light microscopy, in about 2–3 hours. Microscopy-Tutor is based on an accurate, animated, three-dimensional solid model of the microscope, which the user can manipulate. The program includes sections on key concepts, microscope anatomy, Kohler illumination and principles of optics. Essential details like specimen contrast, cover slips and cleaning objectives are also covered. Lastly, there is a final examination consisting of 20 questions with detailed explanations for each answer. The cost is US\$19.

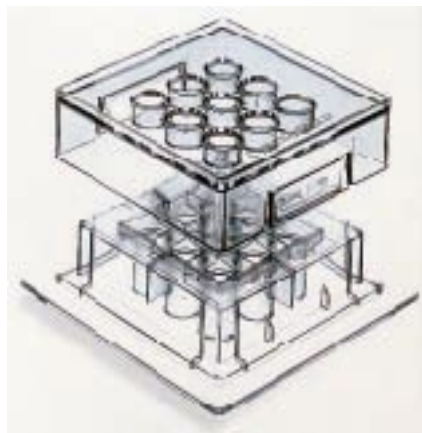
Reader Enquiry No. 105

Cytomorph-b

From Nerbe Plus

Microtest plate for phase-contrast microscopy

Owing to the meniscus that arises at the surface of fluids in small-sized tubes, phase contrast imaging cannot be easily applied to conventional microtest plates. Cytomorph-b overcomes the lens-effect of the meniscus to allow morphological examination of unstained living cells using phase-contrast



Cytomorph-b 3 × 3 microplates (with cover).



Miniscusless — phase-contrast images with Cytomorph-b (right) and without (left).

techniques. Cytomorph-b is a 3 × 3-well flat-bottomed tissue-culture-compatible (TC-treated) microtest plate for morphological, biochemical and immunochemical investigations. Each well measures 5 mm in diameter and leads at the top into an overflow space with a square cross-section. The 3 × 3 lid has flat-bottomed hollow feet congruent to the wells of the plate. It can be locked at different heights: the upper ventilation ensures gas exchange and the lower microscopy position permits the feet of the lid to come into contact with the liquid in the wells. This flattens the meniscus of the liquid surface and prevents the lens effect. Phase-contrast microscopy, as well as photometrical, multi-point measurements, are carried out in the microscopy position. Bright-field microscopy and central single-point measurements are possible with both lid positions. An adapter makes the microplate compatible with various microplate readers.

Reader Enquiry No. 106

DMLFS microscope

From Leica

A fixed-stage microscope imaging system

Designed for ultraviolet (340-nm) fluorescence excitation, and visible and near-infrared (NIR) imaging of live cells, this microscope utilizes a precision focusing nosepiece for fixed stages. Providing a highly stable platform for patch-clamp research techniques, the DM LFS offers the ability to use a wide variety of application-specific, stage-platform and x-y staging solutions. Micromanipulators holding micropipettes and microelectrode probe tips are securely mounted on isolated and fixed platforms, whereas the microscope nosepiece focuses and is moved in the x and y directions over the micromanipulator platform, as is the entire microscope itself. A multi-turreted, sealed condenser provides drainage in the event of media spillage from the drainage area. The system offers a visible/IR-corrected observation tube and a low-cost NIR imaging beam splitter with dual-imaging port and IR dichromatic mirror, allowing simultaneous imaging of 100 per cent visible and 100 per cent IR portions of the spectrum. New long working dis-

tance, water-immersion HCX APO L U-V-I objectives provide a single set of objectives that allow high-performance imaging from 340-nm ultraviolet to visible to 1,000-nm NIR. Now a single-objective set allows optimal imaging and/or quantification of Ca^{2+} with 340-nm excitation, as well as near IR optimized, interference contrast for live cell observations through thick or opaque sections of tissue.

Reader Enquiry No. 107

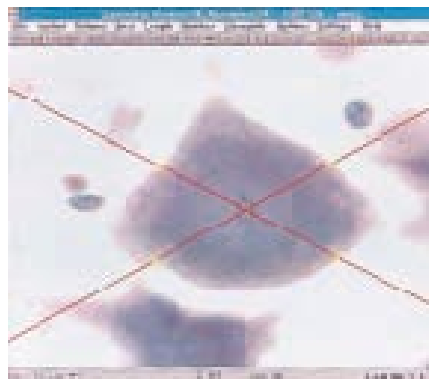
C.A.S.T. Grid software

From Olympus

A system for three-dimensional quantification using the company's BX systems

The system simplifies the use of stereological methods on tissue samples by combining an Olympus BX50 microscope with software developed in cooperation with the Stereological Research Laboratory in Aarhus and the International Stereology Centre at Olympus Denmark. The system uses a motorized stage for automatic sampling, a microcator to monitor the focal plane, a SVHS video camera, a standard PC and a monitor. With training and application support from the International Stereology Centre, users can obtain reliable quantitative estimates by employing stereological methods that are said to greatly reduce sampling time. For example, it is possible to carry out a full neuron count on a complete brain in one day; previously, a brain neuron count of one brain might take up to six months, says Olympus. A variety of multi-dimensional probes can be used to provide information on specimen characteristics, including volumes, surface areas, lengths and numbers of particles or cells, as well as several more advanced estimates. The software allows for numerous grid types to be superimposed onto a live video image to provide uniform sampling and collect data for global estimators. Grid types include points, unbiased counting frames, line segments, lines, cycloids and spatial distribution grids.

Reader Enquiry No. 108



C.A.S.T. Grid software for cellular quantification.

Image analysis systems**LAS-1000**

From Fuji Photo Film

A luminescent image analysis system for use in biochemistry and molecular biology

With an ultrasensitive cooled CCD camera, this quantitative image analysis system captures the faint light image of the two-dimensional distribution of DNA or protein fragments labelled with a chemiluminescent reagent. Additionally, the system can capture fluorescent images using an optional epi-illuminator with safe, blue LEDs. This system has been developed to avoid the hazards of using radioisotopes. Like Fuji's earlier radioisotope-based systems, this fluorescence technology utilizes a 2.54-cm CCD chip with on-chip micro lenses (1.3 million pixels (1,384 × 922), 11 × 11 µm per pixel). The system uses electronic cooling in tandem with forced-air cooling to achieve an operating temperature of 130 °C. The dynamic range is stated to cover four orders of magnitude. The hardware is Macintosh OS and Windows95 compatible.

Reader Enquiry No. 109**Ultra-Imager-5000**

From Ultra-Lum

Gel documentation and image analysis system

This dual-light, mid-sized transilluminator is 25.4 cm wide, has a compact low-profile darkroom cabinet, an integral CCD camera with a motorized zoom lens, a 256 grey-scale thermal printer and a PCI frame grabber (Pentium or PowerMac compatible). There is also Image PC image processing and analysis software, as well as a new Ultra-Glo ultraviolet/visible light converter. The converter allows imaging of visible-stained gels on ultraviolet transilluminators. This fluorescently dyed acrylic polymer glows bright orange under ultraviolet illumination, appearing white to monochrome cameras. Because Ultra-Glo also diffuses light, it provides uniform illumination and eliminates uneven shadowing problems inherent in most white-light illuminators. With the converter, the system is said to combine two ultraviolet wavelengths with visible light on the same filter surface (up to 21 cm × 25 cm) under one small cabinet. ImagePC software edits and annotates captured gel images directly on screen and performs basic image analysis, including calibrated length, density measurements and colony counting. With this program, users can produce publication-quality prints, archive images to disk, and import them into optional analysis software for more complex analysis.

Reader Enquiry No. 110**It's a snap with Polaroid's gel analysis system.****Gel analysis kit**

From Polaroid and Media Cybernetics

Electrophoresis gel analysis kit to record, digitize and analyse electrophoresis gels

The kit includes Polaroid's GelCam camera, PhotoPad digital scanner and Media Cybernetics' Gel-Pro analysis software. Together, these are said to create an affordable, accurate and fully integrated solution for quantifying gels. The GelCam camera is optimized for gel images — simply place the hood over the gel and press the shutter button. The light-tight hood allows the user to expose in room light without using a darkroom and the instant prints provide a finished picture in less than a minute. The PhotoPad allows the user to digitize new and existing gel photographs with push-button ease. The photograph is drawn into the scanner to produce a digital file with 256 grey levels and up to 400 d.p.i. resolution. The PhotoPad can also produce 24-bit colour files, if scans of colour photographs are needed for other applications. Gel-Pro analyser performs all standard laboratory analysis routines quickly and accurately. The image processing software automatically identifies lanes, determines band locations, measures optical density or emitted fluorescence and calculates relative mass and molecular weight. The kit exports information to other Windows programs, including word processing, spreadsheet and presentation software.

Reader Enquiry No. 111**AlphaEase 3.3**

From Alpha Innotech

Image-enhancement software for both good and poor culture images

Expanded functions help users to detect colonies that are hard to see visually. Complete digital contrast control of black, white and gamma increases the ease of counting. Included in the package are manual and automatic colony counting functions, as well as the ability to overlay for subtraction of replicate images. According to the manufacturer, it takes just seconds to count two different colour colonies simultaneously. These enhanced digital images can be saved and used in document processing programs,

or printed on 5-s thermal, photographic-quality paper for notebook and file storage. Also included are annotation and image analysis functions. The program requires SoftWindows for PowerMacs, Windows 3.1 or Windows95, and 8 MB of RAM.

Reader Enquiry No. 112**Shakers and incubation****RS6000**

From Stem

A heated agitator station for drug discovery and biotechnology

This station is designed to help chemists and biotechnologists to synthesize compounds easily and inexpensively. Applications include drug discovery, combinatorial chemistry, organic synthesis and process development. Precise control reactions or incubations are performed in a removable reaction block, which fits securely into a heated platform. This then rotates in a circular orbit to give efficient mixing of the reactants. A 'soft-start' mechanism is used to begin agitation, minimizing sample damage. Agitation is then increased up to full vortex. The temperature can be adjusted from ambient to 150 °C. In addition, there are also customizable reaction blocks, which are currently available to hold 96 × 16-mm test tubes, chemically resistant PTFE microtitre plates, HiTops manifolds from Polyfiltronics and Calypso plates from Charybdis. Moreover, it is said to take just moments to set up manual or robotic operation — setting the required temperature and agitation speed also takes just a few moments using the simple front panel controls. A clear digital readout of actual temperature and r.p.m. is presented on the bright backlit liquid crystal display. Full remote control via RS232 or RS485 interfaces is possible giving the additional facilities of unattended operation, speed variation and temperature ramping over user-defined time periods.

Reader Enquiry No. 113**Infors**

From GRI

A range of high-quality shakers, shaking incubators and fermenters for aerobic and anaerobic organisms

These laboratory appliances allow high speeds of rotation to ensure high rates of gas exchange, while remaining both vibration-free and silent running. Shakers range from the Labotron miniature rotary shaker, a small benchtop model, to the RS-306 pallet-type and RC-406 large-capacity rotary shakers. For those requiring shaking incubators, the benchtop Aquatron and Aerotron incubators offer air or water heat transfer and gas-tight closure. Multitron is said to offer a flexible, high capacity model that can be configured as a free-standing base model or as a master unit with stackable extensions.

The intermediately sized Novotron is a compact benchtop unit. The Sixfors multifermenter system allows six small, independent units to be controlled simultaneously from one processing unit. Labfors is a single or array fermenter — a free-standing benchtop unit that can run independently or run up to six units from a single microprocessor.

Reader Enquiry No. 114

Odds and ends

MMP poster

From Amersham

A new poster that displays current understanding of MMP activation

The new poster summarizes the key areas of wound healing, arthritis, tumour invasion, growth and vascularization, and uterine expression. There is a comprehensive table outlining the key enzyme molecules and their inhibitors with details on substrate and structure. MMP activity is an increasingly researched field because of its vital component in controlling degradation of the extracellular matrix, says Amersham. It is also believed to be important in many disease states, such as cancer and fibrosis, as well as the normal processes of tissue morphogenesis and bone development.

Reader Enquiry No. 115

Aspex

From Asahi Glass

*An *Saccharomyces pombe*-based protein expression system*

According to the manufacturer, the fission yeast *S. pombe* is capable of expressing intracellularly, or into medium, various active proteins, including cytoplasmic, membrane-derived and secreted proteins. Asahi Glass' strain of *S. pombe* has been optimized for reliable expression of cross-species or allogenic proteins with both high yield and high quality. Aspex is said to provide the benefits of an *E. coli* expression system combined with the advantages of eukaryotic expression systems, such as post-translational modification. It produces recombinant proteins in high concentrations, exceeding a stated 50 per cent in the case of recombinant human lipocortin I. Where output is a concern, the system can produce large quantities of proteins compared to insect and animal cell expression systems, as well as secreting various proteins into synthetic media using the signal sequence derived from the *S. pombe* mating pheromone. This system also has the capability to produce recombinant, mitotically stable yeast containing a high copy number of epi- or extra-chromosomal expression vectors (> 200 per cell). Aspex does not use methanol, which is necessary for including protein production in other systems (for example, *Pichia* or *Hansenula*).

Reader Enquiry No. 116

Ion channel modulators

From Current Drugs LW

This ID Research Alert publication is the latest issue in the ion channel modulators series

This monthly service provides a printed bulletin plus fully searchable, cumulative companion CD-ROMs, offering access to a single and comprehensive source of information on ion channel modulators research. According to the manufacturer, data are gathered weekly by experts from patents, scientific literature, meetings, commercial sources and direct company communications. The bulletin provides monthly highlights of developments, reviews, updates on new patents, papers and drug evaluations giving opinions on latest drug developments for ion channel modulators.

Reader Enquiry No. 117

Handiwork

Semperclean MC

From Semperit

A powder-free glove with a multilayer coating, and an inner coating to minimize irritation

A new type of all-round, powder-free protective glove has been designed to overcome problems sometimes encountered with tearing latex gloves, such as allergic reaction or sensitivity. MC anatomical gloves are suited for people working in laboratory and pharmaceutical environments. The glove material is subjected to a leaching process and thorough washing to eliminate proteins and other substances regarded as the main causes of irritation or skin allergies. The material is then treated with a specially developed synthetic inner multilayer-coating (MC). This coating provides a secondary barrier on the inside of the glove, ensuring optimum perspiration absorption and ease of donning, thus eliminating the need for dusting powder. The anatomical shape of the glove is said to ensure a good fit and minimizes fatigue, especially important when the gloves are being worn for long periods. Semperclean MC gloves also have a micro-rough surface that provides a secure wet grip and sensitivity.

Reader Enquiry No. 118

Lab Ambiglove

From Allegiance Healthcare

A 25.4-cm glove for controlled environments

According to the manufacturer, this is a clean, non-powdered latex glove that offers enhanced protection, consistent quality and a comfortable fit. The glove goes through a variety of tests to ensure that all quality requirements have been met and that it exceeds ASTM' D3578-95 standard for



At hand, Semperclean MC powder-free gloves.

physical requirements, says the manufacturer. For added hand and wrist protection, the glove features a beaded cuff that resists rolldown while improving strength and durability, and as this glove is powder-free, the risk of sample contamination from particulates is reduced. Wet or dry, the glove's textured finish provides a secure grip on smooth, slippery items like pipettes and Petri dishes. It also provides excellent tactile sensitivity necessary for delicate applications. This ambidextrous glove is available in four sizes and is packaged in a 100-count dispenser.

Reader Enquiry No. 119

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card

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